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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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**LIGHT UP THE FLY: *DROSOPHILA* AS A NON-SOCIAL MODEL IN INSECT
SOCIOBIOLOGY**

(Thesis format: Integrated Article)

by

Alison Leigh Camiletti

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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ABSTRACT

Eusocial breeding systems are characterized by a reproductive division of labour. For many social taxa, the queen signals her fecundity to her daughters via a pheromone, which renders them sterile. Solitary insects, in contrast, lack social organization and their personal reproduction is not regulated by social cues. Despite these radically different breeding habits between these two taxa, one prediction from sociogenomic theory is that eusocial taxa evolved their complex caste system through co-option of pathways already present in solitary ancestors. In this thesis, I present a series of comparative experiments that provide support for these conserved genes and gene pathways that regulate reproduction in social versus non-social taxa. First, I show that distinctly non-social *Drosophila melanogaster* can respond to a highly social *Apis mellifera* pheromone (QMP) in a manner similar to sterile worker bees – namely, by turning *off* their ovaries and foregoing reproduction. Second, I show that this conspicuous interspecific response is conserved at a genetic level, where the presence of certain *foraging* alleles can elicit variable responses to the pheromone in a manner similar to that in the bee. Third, I suggest that solitary and eusocial species use a conserved olfactory signaling mechanism to elicit reproductive responses to QMP. Using mutant *Drosophila* lines and an RNAi-mediated screen of olfactory receptors, I identify five top receptors as candidates for the perception of QMP and subsequent reduced ovary phenotypes. Lastly, I use *Drosophila* to investigate the functional association between two opposing social cues, royal jelly and QMP and their ability to modulate ovarian development. These results showcase the power of the comparative approach in identifying genes and gene pathways involved in the regulation of worker sterility, and suggest that the genetic basis of characteristically eusocial behaviours like reproductive altruism, are conserved in non-social insects.

Keywords

Drosophila melanogaster; *Apis mellifera*; reproductive altruism; social evolution; comparative analysis; ovary development; queen pheromones

CO-AUTHORSHIP STATEMENT

Chapter 1: On the potential for *Drosophila* as a model behavioural system in sociobiology was written by Alison Leigh Camiletti and Dr. Graham Thompson as a co-author. Dr. Graham Thompson edited the manuscript and provided scientific oversight. I intend to submit this as a manuscript for publication.

Chapter 2: Honey bee queen mandibular pheromone inhibits ovary development and fecundity in a fruit fly was written by Alison Leigh Camiletti with Dr. Graham Thompson and Dr. Anthony Percival-Smith as co-authors. Specifically, Alison Leigh Camiletti conducted all experiments, performed confocal image analysis, performed subsequent statistical analysis on the data, and wrote the manuscript. Dr. Graham Thompson helped to conceive the idea, provided experimental oversight, and edited the manuscript. Dr. Anthony Percival-Smith helped to conceive the idea, provided experimental oversight and approved the manuscript prior to publication.

Chapter 3: How flies respond to honey bee pheromone: the role of the *foraging* gene on reproductive response to queen mandibular pheromone was written by Alison Leigh Camiletti with Dr. Graham Thompson and David N. Awde as co-authors. Specifically, Alison Leigh Camiletti conducted the majority of the experiments, performed confocal image analysis, statistical analysis of the data and wrote the manuscript. David N. Awde assisted in performing experiments and provided editorial comments prior to manuscript publication. Dr. Graham Thompson helped to conceive the idea, provided experimental oversight and edited the manuscript.

Chapter 4: Odorant receptors for honey bee queen mandibular pheromone: a functional genomic screen using *Drosophila* was written by Alison Leigh Camiletti with Dr. Graham Thompson and Dr. Anthony Percival-Smith as co-authors. Specifically, Alison Leigh Camiletti conducted all experiments, performed confocal image analysis, statistical analysis of the data and wrote the manuscript. Dr. Graham Thompson helped to conceive the idea, provided experimental oversight, and edited the manuscript. Dr. Anthony Percival-Smith helped to conceive the idea and provided experimental oversight. I intend to submit this as a manuscript for publication.

Chapter 5: Honey bee royal jelly and queen pheromone as manipulative agents in fly reproduction was written by Alison Leigh Camiletti. Alison Leigh Camiletti conducted all experiments, performed confocal image analysis, statistical analysis of the data and wrote the manuscript. Dr. Anthony Percival-Smith and Dr. Graham Thompson helped to conceive the idea and provided experimental oversight.

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LIST OF ABBREVIATIONS

9-HDA: 9-hydroxydec-2-enoic acid

9-ODA: (E)-9-oxodec-2-enoic acid

AE: after eclosion

AEL: after egg laying

AmOR: *Apis mellifera* olfactory receptor

Amfor: *Apis mellifera* foraging

ANOVA: analysis of variance

BSC ID: Bloomington Stock Center identification number

CaLexA: calcium-dependent nuclear import of LexA

CI: confidence interval

CRISPER: clustered regularly interspaced short palindromic repeats

DAPI: 4',6-diamidino-2-phenylindole

dcr2: dicer 2

D-PBS: Dulbecco's Phosphate-buffered saline

dsRNA: double stranded RNA

EGFR: epidermal growth factor receptor

for: *foraging*

***for*^R:** *foraging* rover allele

***for*^S:** *foraging* sitter allele

g: Hedge's g

GD: P-element RNA interference library

HOB: Methyl p-hydroxybenzoate)

HVA: 4-hydroxy-3-methoxyphenylethanol or homovanillyl alcohol

IIS: insulin/ insulin-like signaling

JH: juvenile hormone

KK: phiC31 RNA interference library

MCS: maximum common substructure

NFAT: nuclear factor of activated T cells

OR: olfactory receptor

Ore-R: *Oregon-R*

PBT: Phosphate buffered saline with Triton-X

PKG: cGMP-dependent protein kinase

PTEN: phosphatase and tensin

qe: queen equivalent

QMP: queen mandibular pheromone

Ras85D: activated form of Ras oncogene 85D

RGPH: Reproductive Ground Plan Hypothesis

RJ: royal jelly

RNAi: RNA interference

TOR: target of rapamycin

Ts: Tanimoto score

Vn: Vein

Vg: vitellogenin

Chapter 1

1 General Introduction

1.1 On the evolutionary genetic basis of sociobiology

Sociobiology is the evolutionary genetic study of social behaviour (Bourke 2011b; Crozier & Pamilo 1996; Frank 1998). Its premise is simple: natural selection of gene variants can explain evolved aspects of behaviour. By adopting this gene's-eye-view of behavioural evolution we can begin to ask, how does a gene associated with a particular behaviour increase in frequency? For social phenotypes, whereby the trait is defined in terms of fitness consequences (West et al. 2007), this approach has proven particularly useful – for example, by explaining how genes for altruism can evolve despite their cost to personal fitness (Bourke 2014).

Further, by incorporating the notion of environmental selection of genetic variants into its very framework, sociobiology implicitly melds 'nature' with 'nurture' (Crozier 2008) and can advance our understanding of behavioural evolution on two fronts. First, it makes clear that differences in genotype (or environment) can explain individual differences in behaviour, which opens up the prospect of finding the very genes (or environments) involved. Second, it questions the adaptive value of behavioural variants and their relative impact on fitness, which helps us to understand the ultimate function of behaviour. The molecular 'how' combined with the evolutionary 'why' of sociobiology make this field as fascinating as it is contentious (Segerstrale 2000).

At a practical level, sociobiologists have been successful at adopting analytical tools developed within the social sciences – for example, longitudinal or cross-sectional

studies, common garden or cross-fostering techniques, and population versus individual level analyses of variance, etc. – are all commonly used in this field (Hughes 1998). Moreover, sociobiologists have begun to deploy sophisticated molecular tools imported from the health sciences (Smith et al. 2008). These tools include powerful genotype-phenotype association analyses, molecular screens and powerful statistical tests for determining the genetic effects on social traits. The field of sociobiology is therefore burgeoning and has a history of assimilating seemingly disparate angles and ideas into its single unified conceptual framework that is Hamilton's (1964) inclusive fitness theory (Abbot et al. 2011; Gardner et al. 2011; Reeve 2001).

1.2 Sociobiology meets inclusive fitness theory

Inclusive fitness theory lends itself well to the evolutionary genetic study of behaviour. For one, it is explicitly gene centric – literally, Hamilton's seminal paper is entitled *The Genetical Evolution of Social Behaviour* – which makes clear the primacy of gene-level thinking. This emphasis on the gene as the unit of selection has, in the past 50 years, reshaped our understanding of how selection works (Dawkins 1976; Herbers 2013). Specifically, it generalizes the Darwinian understanding that selection tends to maximize the direct fitness of individuals. Strictly speaking, it doesn't. Rather, it tends to maximize the fitness of alleles that, as Hamilton showed, can be transmitted indirectly via reproducing, non-descendent relatives. Darwin's emphasis on direct fitness can therefore be regarded as a special case of Hamilton's more general theory of inclusive fitness, which includes both direct and indirect fitness components.

When fitness is correctly partitioned into its constituent components, it is possible to clearly distinguish four different types of social behaviour (Table 1.1). For example, selfishness is recognized by a positive (direct) fitness effect for an individual actor with a correspondingly negative effect on one or more receivers who are, in effect, exploited. Examples of selfishness are common and include all manner of mate and resource competitions among individuals. Cooperation, by contrast, is a qualitatively different type of social interaction that is recognized from the positive fitness outcome for actor and receiver alike. Selfishness and cooperation are readily explained by positive fitness effects on the actor and do not invoke any indirect fitness effects.

1.2.1 The special case of altruism

Altruism, however, is different. This type of social interaction is costly, by definition, to the actor, and thus cannot evolve via direct benefit. Still, altruism *has* evolved and is most evident in the reproductively altruistic castes of the eusocial insects (Liao et al. 2015). Hamilton's insight has proven especially helpful to explain altruism (Crozier & Crespi 2000), whereby the altruist positively affects the direct fitness of one or more receivers. Altruism is rare in the tree of life (Choe & Crespi 1997) but can evolve under conditions that are specified by Hamilton's Rule. The rule – so named by Charnov (1977) – is a heuristic statement that predicts altruism when the direct cost, c , of helping is small in relation to the direct fitness benefit, b , to the recipient of that help, provided the recipient is sufficiently likely, r , to carry copies of the causal gene. Or, $rb > c$.

In the 50 years since Hamilton clarified Darwin's imperfect understanding of altruism, selfless behaviour is now recognized as having evolved more than 11 times independently on the tree of life, and is especially common among certain orders of

Table 1.1 Direct fitness benefits and costs of behaviours performed by the focal individual, the actor, directed towards another individual, the receiver. Note that altruistic behaviour benefits the receiver at a fitness cost to the actor.

		Fitness effect on receiver	
		Benefit	Cost
Fitness effect on actor	Benefit	<i>Cooperative</i>	<i>Selfish</i>
	Cost	<i>Altruistic</i>	<i>Spiteful</i>

insects (Bourke 2011b) where helper individuals forfeit their own reproduction or even lives to benefit their reproducing relatives. In its extreme form, ants (Jones et al. 2004) and termites (Sobotnik et al. 2012) provide stunning examples of altruism; defensive castes will simply explode (via the contraction of glands in their heads) to repel invaders, and in so doing promote the evolution of 'exploding' among their surviving kin. Outside of the insects, kamakazi and other notable forms of altruism are emerging from the study of microorganisms like bacteria (Ackermann et al. 2008; Kreft 2004) and fungi (Queller et al. 2003), where self-sacrificing cells can assist reproducing relatives to the exclusion of unrelated cells that lack the causal gene.

1.3 Genes for altruism

The independent evolution of altruism across different spectra of life creates opportunities for comparative analyses (Crespi 1996). On one hand, it is possible that each origin is a unique response to indirect selection on taxon-specific genes 'for' altruism. If so, then we expect to see little homology between species for gene sets that regulate altruistic traits. On the other hand, it is conceivable that the evolution and expression of altruism occasionally or regularly involves conserved genes (Robinson & Ben-Shahar 2002) or other shared genetic features, like pathways (Mullen et al. 2014) or linkage groups (Lattorff & Moritz 2013). To the extent that conserved genes were repeatedly co-opted to regulate social traits, a major prediction from the 'evo-devo' theme as applied to sociobiology (Toth & Robinson 2007), we can exploit comparative analyses to find them.

However, finding these genes may not be easy. As insightful as inclusive fitness theory is at highlighting the importance of genes enabling the evolution of social traits, the identity

of the genes themselves remains mostly unknown. In my view, there is now a real opportunity to bring together the evolutionary ideas of sociobiology with the practical tools of molecular biology. Molecular biology is well equipped to find genes associated with any number of traits, but so-far the application of powerful gene-finding tools to sociobiology has not been widely adopted. In principle, it should be possible to find genes that underlie altruism. Why not? Reproductive altruism has evolved, and as such, must be underlain by genes that have been selected to regulate these behaviours within social environments.

In this practical sense, genes for altruism are not just hypothetical variables in a conceptual formula - as in Hamilton's 'g' (Hamilton 1963). They are real molecular loci, even if they masquerade as relatively 'normal' genes with unspectacular metabolic, structural, nervous, developmental or regulatory functions (Bloch & Grozinger 2011; Mullen & Thompson 2015). In effect, a gene for altruism, or, a gene for *any* social trait, can be detected by the genotypic difference that explains the behavioural variant in a given environment – for example, a genotypic tendency for parental (selfish) versus alloparental (altruistic) care. For sociobiology this basic tenet of behavioural genetics should be readily applied through Hamilton's rule, which nicely phrases the conditional effect of genetic differences within an environmental context. This prediction from sociobiology, that altruism will evolve when $rb > c$, is useful for testing the conditions under which altruism, or alternatively, cooperation or spite, can evolve. If so, it should be possible to find the genes under selection that mediate the expression of social behaviour. So where are these genes?

1.4 On the potential for finding genes associated with insect sociality

Eusocial insects offer a study of reproductive altruism in its most extreme form. Within advanced eusocial colonies, the presence of functionally sterile castes that forego their own reproduction to help their queen mother rear additional offspring is a textbook example of reproductive altruism (e.g., Alcock 2013), and therefore must have evolved through indirect fitness effects. The direct fitness cost of sterility is presumably compensated when genes 'for' sterility are passed on through her reproducing mother (queen), sisters (future queens) and brothers (drones). Of course, the causal gene is not *selected* if its transmission is incidental, but rather is selected when the *effect* of sterility itself is to increase the direct fitness of related beneficiaries, over and above what their fitness would have been.

What qualities might real genes for altruism have? For one, we expect them to be differentially expressed between reproducing (selfish) and non-reproducing (altruistic) individuals. That is, genes for altruism in the form of sterility should be tuned 'ON' in the altruistic workers but 'OFF' in the reproductives, otherwise the gene would simply go extinct (Bourke 2011a; Queller & Strassmann 1998). One straightforward approach to finding these genes then is to simply contrast gene expression between reproductive and non-reproductive castes, as has been attempted with the honey bee *Apis mellifera* (Mullen et al. 2014).

For this highly eusocial species, reproductive division of labour is maintained by the queen who signals her reproductive maturity through pheromones (Hoover et al. 2003). Workers respond to this honest signal by divesting from their own egg-laying

opportunities, and investing into the queen's (Kocher & Grozinger 2011). The response to queen pheromone is evident upon examination of the worker's reproductive physiology: in the presence of a fecund queen, worker ovaries regress to a few ovarioles and are considered functionally sterile (Velthuis 1970). This sterility is, however, conditional. When the reproductive health of the queen declines, workers are able to re-activate their ovaries and produce some haploid (unfertilized) male-destined offspring (Page & Robinson 1994). Worker sterility is therefore conditional on the social circumstance of the colony, as communicated by queen and brood pheromone (Hoover et al. 2003). Because the pheromonal regulation of honey bee division of labour is well understood (Jarriault & Mercer 2012), it is possible to experimentally manipulate the application of queen or brood pheromone, and with it activation or de-activation of worker ovaries (Backx et al. 2012). From here, one can screen for genes involved.

With the advent of molecular genetic resources that allow for molecular screens from social insects, we rapidly began to uncover single genes or suites of genes that correlate in their expression with worker ovary de-activation. For example, intriguing genes like *major royal jelly proteins*, which are synthesized by workers, and incorporated into royal jelly and subsequently fed to developing brood (Drapeau et al. 2006) are differentially expressed between ovary-active and ovary non-active workers (Thompson et al 2006). Likewise, *vitellogenin* (*Vg*), a yolk protein pre-cursor that is clearly related to reproduction and parental care (Bownes 1982), and that has been co-opted during social evolution for incorporation into brood food (Amdam et al. 2003), is also differentially expressed between ovary-active and ovary non-active workers (Thompson et al 2008).

1.5 On the potential for finding pathways associated with insect sociality

Beyond single genes and genes families, the era of microarrays implicated the insulin/insulin-like signaling (IIS) pathway (Grozinger et al. 2007; Mullen et al. 2014), the target of rapamycin (TOR) pathway (Cardoen et al. 2011), the PIWI RNA pathway (Fischman et al. 2011), the ecdysteroid signaling pathway (Cardoen et al. 2011), the dopamine signaling pathway (Beggs et al. 2007; Oxley et al. 2008), as well as gene sets involved in histone and DNA methylation (Mullen et al. 2014) to be differentially expressed as a function of worker sterility. The technology-driven generation of these candidate genes and pathways was an important step towards understanding the molecular basis of honey bee worker sterility (Mullen & Thompson 2015).

But how do these molecular components fit together to coordinate reproductive decisions within individuals? We expect genetic effects to be mediated through environmentally-responsive networks (Bendesky & Bargmann 2011; Schwander et al. 2010), and indeed, molecular biologists predicted these networks from the honey bee and other social insects (Abbot et al. 2011; Cardoen et al. 2011; Thompson et al. 2008). Insulin signaling and other multi-gene pathways with broader biological functions were among the first to be implicated in the reproductive regulation of social insects, perhaps because these pathways are involved in processes related to nutrition (e.g., glycolysis), immunity (e.g., TOLL pathway) or reproduction, all of which are relevant to the social coordination of colonies (Fischman et al. 2011). Some of these ideas are substantiated by empirical studies that directly estimate the genetic composition of regulatory pathways in worker honey bees (Cardoen et al. 2011; Chandrasekaran et al. 2011; Grozinger & Robinson

2007; Mullen et al. 2014). Multi-gene network studies are useful because they provide systematic molecular hypotheses about how sociality is coordinated at the molecular level. The validity of these hypotheses have, however, yet to be fully tested *in vivo*, probably because it is not yet feasible to perturb networks via gene knock-down in the honey bee, as it is for some other insect model taxa.

1.6 The need for a fully tractable genetic model in insect sociobiology

Although social insects provide an array of complex behavioural phenotypes to study, their feasibility as a model organism is sometimes limited by the complexities of rearing lab colonies that can be perpetuated with controlled breeding. For the social Hymenoptera (ants, social wasps and bees) the haplodiploid mode of sex determination can impose a heavy genetic load on inbreeding, resulting in the production of unproductive diploid males (Beye et al. 2003). Many of the candidate genes and gene pathways for social processes have been identified through association and correlational studies, while functional genomic tests are still in their infancy (Lattorff & Moritz 2013). For example, RNAi analysis is a powerful tool to study phenotypic effects associated with specific gene knockdowns, but this technology is only weakly effective on *Apis* (Jarosch & Moritz 2011; Scott et al. 2013).

Despite this limitations, it may be possible to make crude tests of the most important predictions using other, non-social models as surrogates in sociobiology. Just as the field of evolutionary developmental genetics showed how the same genes were repeatedly co-opted to solve similar problems in phylogenetically distant taxa - e.g., genes related to body segmentation or eye development (Carroll et al. 2001) - sociobiology may too

benefit from a similarly broad comparative approach (Toth & Robinson 2007).

Specifically, we expect that some of the genes involved in reproductive regulation in social taxa will be homologous to those that coordinate reproduction within non-social, solitary insects.

1.7 Reproductive homology between solitary and social taxa

The ovarian ground plan hypothesis (West-Eberhard, 1996; Figure 1.1) captures this idea - namely, that queen-worker specialization arose via disruptive selection on gene networks that once regulated the reproductive and non-reproductive stages of solitary life cycles (Amdam et al. 2006; West-Eberhard 1996). These differences between solitary and eusocial reproductive life cycles are further exemplified in Figure 1.2, that depicts the differences in behaviour and development of the reproductive (queen and drones) and non-reproductive (worker) castes of *Apis mellifera* (A), and that of a reproductive solitary *Drosophila melanogaster* female (B). The idea that genes regulating reproduction are conserved in function across phylogenetically distant lineages has been extended and modified to explain a number of related polyphenisms (Oldroyd & Beekman 2008), including the nurse-to-foraging transition in worker honey bees, and the pollen-vs-nectar preference among foragers. For conserved genes to be repeatedly co-opted during social evolution likely involved new gene regulation rather than new genes *per se* (Robinson & Ben-Shahar 2002).

To juxtapose the idea of genetic conservation, other authors have highlighted a role for genetic novelty in social evolution (Sumner 2014). Consider that new genes, not present

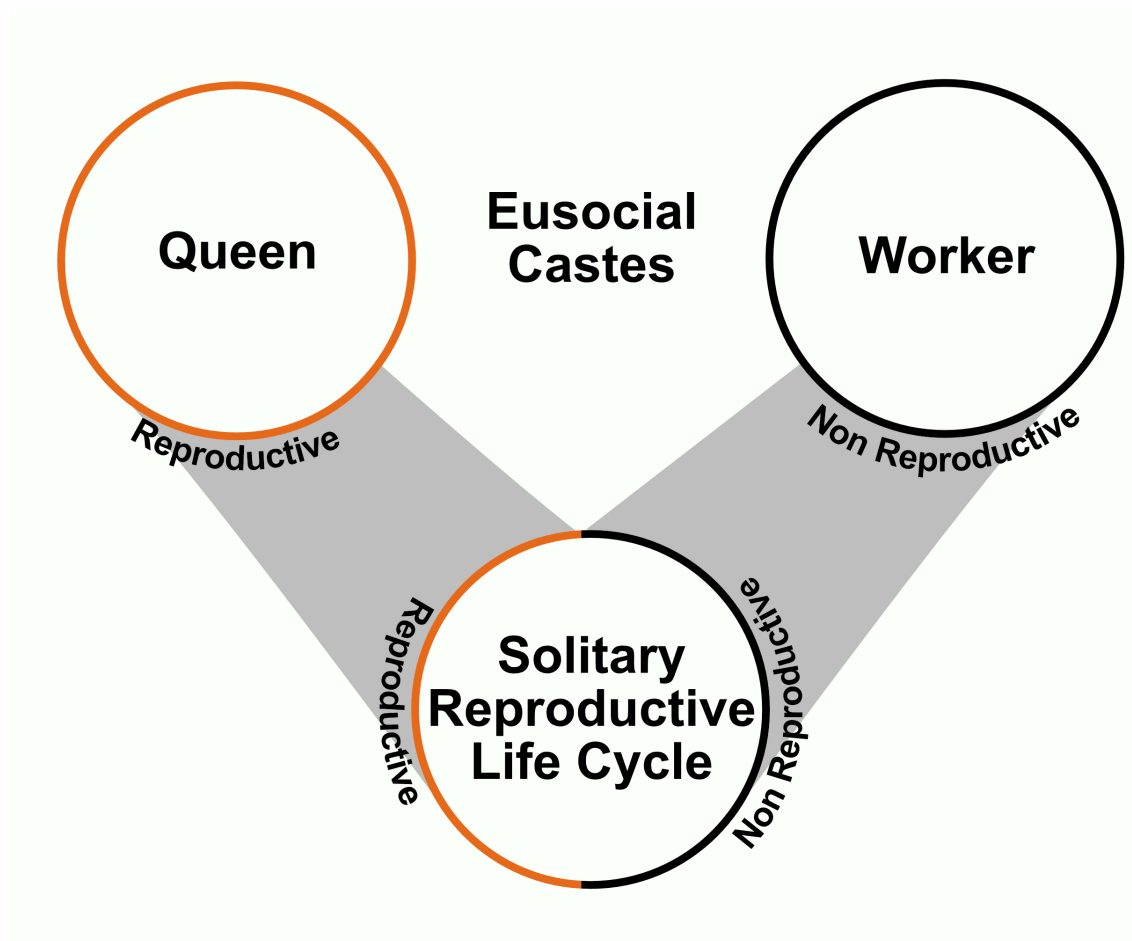
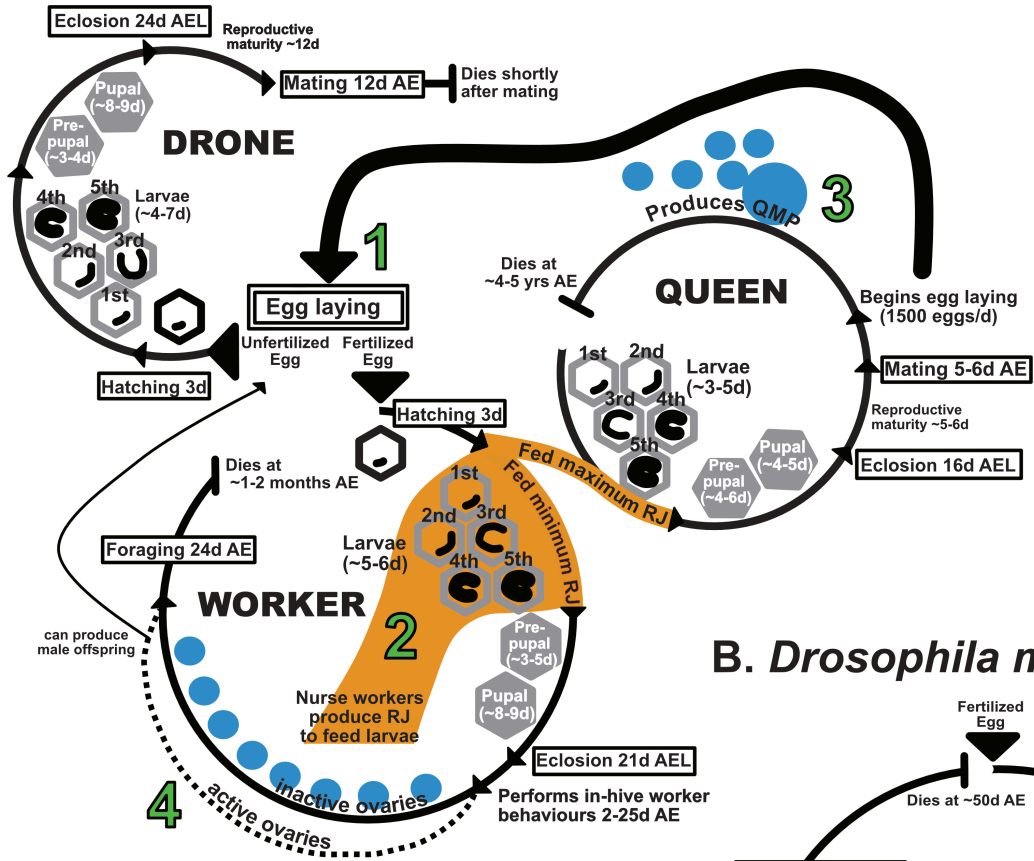


Figure 1.1 Diagram of the ovarian ground plan hypothesis proposed by West-Eberhard (1996). The solitary ancestor of the eusocial Hymenoptera, including *Apis mellifera*, manifested a single phenotype that encompassed the full complement of maternal behaviours, from a reproductive phase that included mating and egg laying and a non-reproductive phase that included foraging, nest building and brood care. It is thought that these two phases of the solitary life cycle became uncoupled through divergent selection that increasingly favored reproductive and non-reproductive specialists. In *Apis mellifera*, these two phases now represent two distinct eusocial castes; the queen that monopolizes the reproductive phase, and the worker that monopolizes the non-reproductive phase.

A. *Apis mellifera*



B. *Drosophila melanogaster*

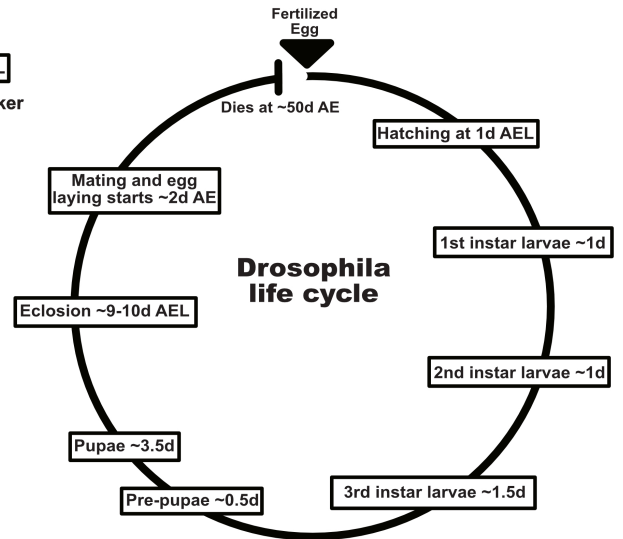


Figure 1.2 Behavioural and developmental comparisons of a social and non-social insect.

*Life-cycle and caste development of *Apis mellifera* (A).* Queens produce females (queen's or workers) and males (drones) by laying either fertilized or unfertilized eggs (1) in empty comb cells within the hive. Female larvae that are fed a diet rich in royal jelly (RJ) develop into queens, while those fed a minimum amount develop into workers (2). This nutritional advantage allows queens to become highly fecund and reproductively dominant. After mating with drones, they lay on average 1500 eggs per day. The queen's fecundity is correlated with her production of queen mandibular pheromone (QMP; 3). This pheromone serves as an honest signal of the queen's egg laying potential and induces worker sterility; whereby, worker ovary development is suppressed, termed ovary inactivation (4). Workers instead engage in non-reproductive tasks, such as hive maintenance, defense activities, and feeding the larval brood with the RJ they produce. Young in-hive workers are highly sensitive to changes in QMP and can re-activate their ovaries in instances of queen death, queen absence or declines in queen fertility. Workers with active ovaries do not mate yet can lay unfertilized eggs that develop into drones.

*Life-cycle of *Drosophila melanogaster* females (B).* Larval development in *Drosophila* is shorter than that of honey bees, consisting of 3 larval instars, and ~4 days of pupation. Adult females mate and begin egg laying rather quickly after emergence and lay on average 50-70 eggs per day. Day (d), after eclosion (AE), after egg laying (AEL), years (yrs). *Adapted from:* Winston, M.L. (1987) *The biology of the honey bee*. Harvard University Press, Cambridge; and *Biology of *Drosophila** (1994). M. Demerec ed. Cold Spring Harbour Laboratory Press, New York.

in solitary ancestors, now regulate the expression of social traits (Johnson & Linksvayer 2010). Which is it? New genes, or new gene regulation? The latest compromise suggests a mix of these two sources of variation, whereby conserved genes and even pathways are regulated in new and novel ways to give rise to social traits (Berens et al. 2015; Mikheyev & Linksvayer 2015). This ‘mosaic’ view of social evolution may best explain the pattern of shared and derived features, across the social Hymenoptera anyway (Rehan & Toth 2015).

1.8 Comparative analyses within a phylogenetic context

1.8.1 *Apis* versus other social Hymenoptera

Comparisons between *Apis spp.* and other bee and hymenopteran insects can be insightful (Michener 1974). Each degree of social complexity is defined by the presence of three conspicuous characters: cooperative brood care, reproductive division of labour and an overlap of adult generations capable of contributing to reproduction. Genomic comparisons between species that differ in social structure may therefore help to highlight the genes involved in each social transition (Schwarz et al. 2007). Comparisons between ants and honeybees have revealed that the origins of social behaviour are likely rooted in the differential expression of conserved (Simola et al. 2013) over derived gene sets. For example, similar genes involved in pathways related to development and metabolism regulate worker caste differences in different species of ants (Mikheyev & Linksvayer 2015), as they do in *Apis* (Mutti et al. 2011). In support of the ovarian ground plan hypothesis, reproductive genes like *vitellogenin* modulate caste differentiation in social species like bees and ants. For example, *Vg* is highly duplicated in ant species with paralogues showing different caste specific expression patterns (Morandin et al. 2014).

Divergence within the subfamily Apinae alone has produced tribes of bees that retained their solitary ancestry (Euglossini), those that exhibit a range of sociality from solitary to eusocial (Allodapini and Halictini) and those that became advanced eusocial (Apini and Meliponini). Secondly, within the order Hymenoptera (ants, bees, wasps, and sawflies) eusociality has evolved eleven times independently (Hughes et al. 2008; Johnson et al. 2013) four times within bees alone (Kocher & Paxton 2014). Large-scale comparisons across several bee species with varying levels of social structure, again found that changes in gene regulation is a key feature of social evolution (Kapheim et al. 2015). As is the case for other social insects, genes comprising conserved pathways such as those involved in neuronal signaling and metabolic processes like carbohydrate metabolism appear to have been positively selected in highly eusocial lineages (Woodard et al. 2011). Interestingly, genes involved in metabolic processes can also regulate solitary and social behavioural states within single species like the sweat bee, *Lasioglossum albipes*, that exhibits flexible social behaviour according to geographic location (Kocher et al. 2013). These examples highlight that these independent evolutionary events are due to regulatory changes of conserved genes and gene pathways (Graham et al. 2011).

1.9 *Apis* versus social non-Hymenoptera

Outside of the Hymenoptera, other insects like termites (Isoptera; Noirot 1990), some species of beetles (Coleoptera; Kent & Simpson 1992), aphids (Aoki 1977), and thirps (Crespi 1992) are considered eusocial by their reproductive division of labour and presence of a non-reproductive defensive caste. Studies on the termite *Cryptotermes secundus* provide evidence that similar genes involved in honey bee reproductive regulation are up-regulated in female neotenic (imature) reproductives. One gene,

Neofem4, is involved in juvenile hormone (JH) regulation, and another, *Neofem3*, is related to Vg (Weil et al. 2007). Division of reproductive labor is also found in non insect arthropods, including sponge-dwelling shrimp (Duffy & Martin 2007), and even in a non-arthropod, like the naked mole rat (Rodentia; Jarvis & Bennett 1993). Investigations of the Damaraland (*Fukomys damarensis*) mole rat found that non-reproductives had suppressed levels of key mammalian reproductive regulators like luteinizing hormone and gonadotropin releasing hormone (Bennett 2011), again highlighting that key modulators of solitary reproductive pathways can be co-opted to regulate eusocial sterility. Comparisons between *Apis* and other insects within Hymenoptera, question the ecological costs and benefits associated with transitions to eusociality, what was the role of genetic factors like relatedness, and further what were the behavioural dynamics within societies that allowed for social transitions to take place (Crespi 1996).

1.9.1 *Apis* versus *Drosophila*

Despite nearly 350 million years of divergence between flies (Diptera) and the order containing bees (Hymenoptera) (Kazemian et al. 2014), comparative studies using the non-social fly as a model of pre-social evolution is an attractive route to gene discovery. Firstly, their established lab culturing and international development of molecular resources associated with '*Drosophila*' biology are enormous (Ashburner & Bergman 2005). Social bees have also joined the post-genomics era (Kapheim et al. 2015; Woodard et al. 2011); whereby, *Apis mellifera* (Honeybee Genome Sequencing Consortium 2006) and five other bee species have been sequenced, three of them just this year, *Apis cerana* (Park et al. 2015), *Bombus terrestris* and *Bombus impatiens* (Sadd & Barribeau 2015). Further, comparative initiatives like iK5

(<http://www.arthropodgenomes.org/wiki/i5K>), suggest that soon many more social genomes will be available for comparative analysis.

Admittedly, *Drosophila* is not a traditional model for sociogenomic research. Even though fruit flies interact with other individuals in the population for feeding (Wu et al. 2003) and mating (Villella & Hall 2008) they lack the behavioural characteristics (i.e., reproductive division of labour) that define them as truly social in sociobiological terms. Despite not having a rich social behavioural repertoire, we can, nonetheless, exploit *Drosophila*'s powerful genomic resources for novel comparative approaches. Table 1.2 provides a summary of the gene finding and gene manipulation techniques currently available for *Drosophila* versus *Apis*. Firstly, we can utilize *Drosophila* mutant lines to test if genes implicated in *Apis* for the regulation of social traits, can regulate similar behaviours in *Drosophila*. For example, do the same genes regulate pheromone perception and ovary inactivation in both *Apis* and *Drosophila*? Secondly, powerful *Drosophila* techniques, such as the Gal4/UAS system, can allow for targeted control of gene expression (Duffy 2002). This technique is especially useful to alter the expression of genes that are involved in conserved pathways of interest in *Apis*, such as those involved in queen-worker caste dimorphism. Using the Gal4/UAS system we can alter gene expression of *Apis* homologs in *Drosophila*, making flies more queen or worker-like, and compare the resulting behavioural changes with those seen in the bee. Third, the Gal4/UAS system can be coupled with techniques such as RNAi, to knock-down gene expression (Bakal 2011). This approach is particularly useful for conducting large screens to determine which sets of genes are associated with a particular phenotype. For comparative approaches, these screens can be initiated and performed in *Drosophila*,

Table 1.2 A comparison of features associated with laboratory analysis, genetic structure and manipulation available for *Apis mellifera* versus *Drosophila melanogaster*.

	<i>Apis mellifera</i>	<i>Drosophila melanogaster</i>
Laboratory Analysis		
<i>Rearing</i>	Require social interaction for optimal development	Can be raised in social isolation
<i>Containment</i>	Observation hive for outdoor foraging of high quality pollen sources	Small portable plastic vials that contain an agar based food source
<i>Study period</i>	Bees overwinter and lay eggs in early spring	Can be maintained year round in the laboratory
<i>Breeding Populations</i>	Single queen (1500 eggs per day) and a few drones	All females and males are fully fertile (50-70 eggs per day)
<i>Population effects</i>	Colony composition, population density, humidity/ temperature or seasonal effects	Population density, humidity/ temperature in laboratory strains
<i>Developmental time</i>	Colony requires 8 weeks to attain sufficient number of foragers. Queens ~16 days, workers ~21 days, and drones ~24 days from egg laying.	~9-10 days from egg laying to eclosion
<i>Lifespan</i>	Queen ~ 4-5 yrs; workers ~1-2 months during summer, ~4-9 months during winter; drones ~12-14 days if mating, can live up to ~4 months	~50 days after emergence

<i>Selective Breeding</i>	Controlled mating requires artificial insemination of queen, or geographical isolation/ temporal control over queen mating flights	Virgin females are easily mated to specific males.
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Genetic Structure

<i>Genome size</i>	236Mb (16 chromosomes)	130Mb (4 chromosomes)
<i>Ploidy</i>	Haplodiploid males and diploid females	Diploid
<i>Sex determination</i>	Haplodiploid sex determination system, <i>csd</i> gene	Ratio of X chromosomes to autosomes
<i>Recombination</i>	Only in females	Only in females

Genetic Manipulation

<i>Introduction of genetic elements</i>	Feeding and injection	Feeding, injection mating between stable transgenic lines
<i>Inbreeding</i>	Reduction in maintenance of mutant lines	Balancer chromosomes allow for propagation of recessive lethal mutations
<i>Maintenance of mutant lines</i>	Not available	Available
<i>Popular Genetic Tools</i>		
P-elements	Newly available <i>piggyBac</i>	~19,000 available to affect gene function
RNAi	Available but limited due to tissue specificity of dsRNAi uptake	~22,000 lines containing small interfering RNAs disrupt gene function in most tissue types
GAL4/UAS	Not available	Widely used

Genetic Resources

Genetic Databases	Beebase	Flybase
Stock centers	Not available	Bloomington <i>Drosophila</i> Stock Center (BDSC), Vienna <i>Drosophila</i> Resource Center (VDRC) and the Kyoto <i>Drosophila</i> Genetic Resource Center

where RNAi is more established, and used to produce a smaller and more focused subset of genes for further investigation in social taxa. Lastly, categorizing genes of interest into GO categories (cellular component, biological process, molecular function) is useful to define what processes are important for social behaviour in *Apis*, and then to focus experimental efforts on pathways that regulate these processes in *Drosophila*.

The wide use and availability of these genetic tools in *Drosophila* is largely due to the ability to maintain stable transgenic lines in this species. Currently, genetic elements can be introduced in *Apis*, like double stranded RNA (dsRNA) for RNAi, but only through feeding or injection (Scott et al. 2013). Unfortunately these methods of introduction are hindered by tissue specificity of dsRNA uptake (Jarosch and Moritz, 2011). Recently there has been progress towards developing stable transgenic lines, through use of a germ-line targeted transposon called *piggyBac* (Schulte et al. 2014). This could greatly facilitate the implementation of tools like Gal4/UAS for direct use in *Apis* in the future (Ben-Shahar 2014).

1.10 *Drosophila* as a non-social model of social gene discovery

Using *Drosophila* to better understand the behaviour of eusocial species dates back to pioneering studies that found that adult female fruit flies (Sannasi 1969) and house flies (*Musca domestica*; Nayer 1963) had reduced ovary size like queen-right worker bees when exposed to a single component of honeybee queen pheromone. It was further found that this same component can cause reduced ovarian development in other social insect

species such as termites (Hrdý et al. 1960; Sannasi & George 1972) and ants (Carlisle & Butler 1956).

Since then, comparisons of bees and flies have revealed striking degrees of conservation in genes that regulate behaviour (Reaume & Sokolowski 2011). The bee homolog of the *Drosophila foraging* gene, that causes two distinct foraging polymorphs in the fly (Osborne 1997), regulates worker nurse-to-forager transitions (Ben-Shahar et al. 2002). Amazingly, homologs for *foraging* have also been found in a *Pheidole* ant where it regulates foraging behaviour between major and minor worker subcastes (Lucas & Sokolowski 2009). Other hive specific cues such as royalactin, the main component of royal jelly, also elicit similar phenotypes in *Drosophila* (Kamakura 2011). For example, when young larvae are reared in a medium containing royalactin, a bee protein shown to be involved in worker-to-queen transitions, they developed faster and into larger female flies, and like queens have increased fecundity (Kamakura 2011).

Our lab has shown that *Drosophila* males and females have remarkable conservation in response to the various behaviours that queen pheromone elicits in the hive. When exposed to synthetic QMP, wild type *Oregon R* (*Ore-R*) female flies developed smaller ovaries that contained fewer eggs compared to unexposed controls (Chapter 1). Further QMP exposure reduced the number of adult offspring produced after mating (Camiletti et al. 2013). This observation indicates that fruit flies may contain similar conserved genetics pathways as honeybees, allowing them to regulate their ovaries in response to this pheromone. I subsequently found that fruit fly polymorphs of the *foraging* gene, rover and sitters, were differentially responsive to QMP like nurse and worker bees (Chapter 2). Whereby, rovers like foragers were less responsive to QMP, and sitters like

nurses were more responsive (Camiletti et al. 2014). Recently I have extended these studies to uncover the olfactory response to QMP and the receptors responsible for pheromone perception (Chapter 4). To this end I show that wild-type flies are still able to respond to QMP through olfaction alone and that olfactory mutants fail to reduce their ovaries in response to QMP. By screening the majority of the adult olfactory receptors present in *Drosophila* we provide a candidate gene list of receptors that may be responsible for QMP response and discuss their homology to honey bee receptors. Finally, in this thesis I investigate the role of royal jelly in mediating response to QMP, by assessing its ability to modulate response to the pheromone. We suggest that royal jelly may be able to reduce the ovarian effects associated with QMP exposure, and propose that the nature of this interaction may be tissue specific (Chapter 5).

My novel results have spurred a new series of studies in our laboratory. First, undergraduate student Tom Liu has shown that QMP significantly affects male mating intensity in *Drosophila* comparable to its role as a sex pheromone in *Apis* (Liu 2015). Masters student Justin Croft, has since found that males and females are attracted to QMP in behavioural choice assays, a finding that is comparable to this pheromone's ability to initiate retinue responses in worker bees and function as a sexual attractant for males. The fact that a divergent pheromone that has evolved a diversity of functions in *Apis*, can elicit a similar range of homologous behaviours in flies raised the prospect that *Drosophila* can be used in conjunction with honey bee QMP as a model to identify and reconstruct some of the molecular machinery that regulates reproductive divisions in labour within honey bee and potentially other social insect societies.

In conclusion, inclusive fitness theory is conceptually appealing, but its application towards finding genes associated with social traits, has been slow. Advances in the genomic sequencing of more social taxa and the development of new molecular tools will serve to significantly advance social gene discovery. In the mean time, comparative approaches with more well established model organisms like *Drosophila*, can be used to support theoretical models like the ovarian ground plan hypothesis that serve to address the evolutionary origins of social behaviour. Further these studies can provide molecular data towards uncovering conserved gene sets for behaviours central to kin theory, like reproductive altruism. Genes identified from *Drosophila* can then be extended to a broader range of social taxa to reveal a more unifying view of reproductive regulation in insects and help us piece together the molecular underpinnings of inclusive fitness theory.

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Chapter 2

2 Honey bee queen mandibular pheromone inhibits ovary development and fecundity in a fruit fly

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2.1 Introduction

A conspicuous feature of social insect colonies is the division of labour between reproductive and non-reproductive specialists. In one species of honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), this behaviour is regulated at the proximate level by queen pheromones that signal queen fecundity to her workers (Le Conte & Hefetz 2008). If queen fecundity is sufficient to promote worker reproductive altruism (Hamilton 1964), then workers will generally refrain from activating their ovaries. As a consequence, queenright worker honey bees (i.e., workers in the presence of a queen) are generally considered to be functionally sterile. Although the adaptive significance of sterility is well understood in the context of honey bee biology (Oldroyd & Thompson 2007), the molecular mechanism through which pheromones inhibit ovaries is less clear (Bloch & Grozinger 2011).

One idea to emerge from recent advances in insect sociobiology is that pathways that govern reproductive divisions of labour in social taxa likely evolved from pathways controlling reproduction in solitary taxa (Toth & Robinson 2007). The ‘groundplan’ hypothesis encompasses this idea to predict that conserved sets of genes may help control ovary activation in both solitary and social insects (West-Eberhard 1989). Thus, although the precise mechanism controlling ovary activation in honey bees is not known (Thompson et al. 2008), the mechanism will most likely be sensitive to social context and involve genes conserved from solitary insects (Bloch & Grozinger 2011).

The conceptual premise for functional conservation of pathways between solitary and eusocial taxa is strong (Fischman et al. 2011; Reaume & Sokolowski 2011). And, where it has been examined, comparative gene expression studies have generally confirmed a degree of conservation between genes involved in reproduction between *Apis* and *Drosophila* (Cardoen et al. 2011; Grozinger et al. 2007; Kamakura 2011; Thompson et al. 2008). The insulin/insulin-like signaling pathway (Grozinger et al. 2007), the TOR pathway (Cardoen et al. 2011), the PIWI RNA pathway (Fischman et al. 2011), and the EGFR signaling pathway (Kamakura 2011), potentially play vital roles. Comparative studies suggest that the mechanism for ovary regulation may share evolutionary origins with pathways present in solitary taxa (Amdam et al. 2006), but the extent to which common cues can trigger any underlying mechanism has rarely been tested.

In this study, I test the extent of reproductive gene homology by simply comparing the reproductive response that a non-social female insect has to the ovary-regulating pheromone of a highly social insect. Specifically, I follow precedent to directly compare one social (*A. mellifera*) and one non-social [*Drosophila melanogaster* Meigen (Diptera: Drosophilidae)] insect (Sannasi 1969). Whereas Sannasi (1969) injected a single component (*E*-9-oxo-2-decenoic acid) of the ovary-regulating queen mandibular pheromone (QMP) to suppress fly ovaries, I deliver a multi-component synthetic mimic of queen pheromone to groups of virgin female flies. The synthetic formulation contains 5 active components (Keeling et al. 2003; Slessor et al. 1988) and is available commercially as a high-fidelity mimic of natural queen pheromone (Contech Enterprises, Vancouver, BC, Canada). Flies can be densely aggregated, but this species lacks parental care and division of labour that characterizes truly social (eusocial) insects. Any specific response from fly ovaries to this treatment would be consistent with the presence of a conserved, functionally responsive pathway. I predict that honey bee QMP will inhibit fly ovaries, consistent with how it functions in worker bees (Backx et al. 2012). Regardless of any direct effect on ovaries per se, I also test whether QMP delivered at an early stage in fly development affects the fly's realized fitness, as inferred from the number of offspring.

2.2 Materials and Methods

2.2.1 Fly rearing

I reared all ‘Oregon R’ flies (OR; Bloomington Stock Center, Bloomington, IN, USA) under standard conditions (25 °C, L12:D12 photoperiod) on a diet that consisted of 15 g of yeast from *Saccharomyces cerevisiae* Hansen (Type II; Sigma-Aldrich Life Sciences, St. Louis, MO, USA), 10 g of granulated agar (EMD Millipore, Darmstadt, Germany), 100 g of cornmeal (Unico, Concord, ON, Canada), 60 g of granulated sugar, and 3.75 g of methyl-*p*-hydroxybenzoate (MP Biomedicals, Santa Ana, CA, USA), all made to 1 l of water (after Elgin & Miller, 1980). From this population, I collected next-generation day-old larvae and transferred them in groups of $n = 30$ into wide vials (28.5 × 95 mm) containing 10 ml of the above fly food. I reared larvae in a total of $n = 37$ vials until the adults emerged ~10 days later.

2.2.2 Treatment with queen pheromone

To assess the effect of QMP on ovary activation, I first exposed groups of $n = 5$ virgin flies to synthetic QMP. Synthetic QMP is soluble in ethanol and can be diluted into ‘queen equivalent’ units (qe units; Pankiw et al., 1996). I diluted a stock mass (500 mg \approx 1 184 qe) of QMP with 100% ethanol to make a dilution series of 3, 6.5, 13, and 26.5 qe treatments (by weight). I initially stored these aliquots at -20 °C, prior to heating them (50 °C in a water bath) to liquid form.

To expose groups of day-old virgins, I created a chamber from a modified 50-ml Falcon tube that was fitted at one cut-open end with a cotton plug, and at the other with filter paper (grade 413; VWR International, Radnor, PA, USA). The filter paper was screwed into place under the plastic cap. Using this chamber, I first saturated the filter paper with a yeast and sugar solution (0.1 g yeast, 0.15 g sugar, in 5 ml of 5% ethanol). I then dispensed 20 μ l of the appropriate QMP solution to the center of the filter paper.

Because I did not know the concentration that would affect fly ovaries, if any, I exposed separate groups of females to one of four treatment groups, or to a control solution containing 100% ethanol in place of QMP – that is, 0 qe. My choice of 3 – 26.5 qe units

QMP is meant to capture an unknown range in effect sizes. These doses are nominally high as prepared within the fly-food medium, but the amount actually consumed by individual flies (within groups of five) is presumably much less. I exposed groups of virgin flies to QMP by simply placing them inside the modified Falcon tubes for 48 h. Finally, to control for the possibility of a pharmacological effect from high-dose pheromone of any type, as opposed to a pathway-specific effect from QMP per se, I exposed a control group of flies to a second pheromone. Specifically, I exposed flies to one low (1 μg in 20 μl of hexane) and one arbitrarily high (80 μg in 20 μl of hexane) dose of 7-tricosene (Cayman Chemical Company, Ann Arbor, MI, USA). This naturally occurring fly pheromone produced by males (Scott 1986) has been shown to increase female receptivity for mating (Grillet et al. 2006), and to prevent male- male courtship (Ferveur & Sureau 1996). It is unrelated to QMP and, to my knowledge, is unrelated to ovary phenotype.

2.2.3 Ovary assay

Following treatment, I anesthetized all flies via CO_2 narcosis and dissected paired ovaries (left and right) using an Olympus S7X7 stereomicroscope (Olympus, Richmond Hill, ON, Canada) fitted with an Olympus SZ2-LGD1 light source. I dissected ovaries in $1\times$ Dulbecco's phosphate-buffered saline ($1\times$ D-PBS; Invitrogen, Carlsbad, CA, USA), and fixed dissected tissue in solution (4% formaldehyde in D-PBS, for 20 min). I then stained each set of dissected and fixed ovaries with DAPI (1:2 000) for 1 h (with shaking). Finally, I mounted each specimen in 7% glycerol in D-PBS.

I scored the level of ovary development by two criteria. First, I counted the mature (stage 14) eggs present in either ovary per fly, according to King (1970). Second, I estimated the combined area (μm^2) of both ovaries from a single fly using the 'count size' macro in IMAGE PRO PLUS 7.0 software (Media Cybernetics, Bethesda, MD, USA). For this image-based analysis, I used the maximum intensity projections of confocal images, as captured using a Zeiss LSM 510 confocal microscope and ZEN LIGHT EDITION software (Carl Zeiss Canada, Toronto, ON, Canada). I generally used analysis of variance (ANOVA) to assess differences in egg number, ovary area, and realized fitness (number of pupae and number of eclosed adults) between pheromone-treated and control groups,

and used logistic regression to assess any differences in survivorship. I performed all statistical analysis using IBM Statistics SPSS 19 (Armonk, NY, USA).

2.2.4 Fecundity assay

To assess fecundity, I treated groups of flies as above, but following the 48-h exposure I transferred individual females into new vials containing 5 ml of fly food. To each tube, I introduced two males age-matched to the females. I left each threesome in a food vial for 24 h. I then removed the adults and left their offspring to mature. As a measure of fecundity, I recorded the number of flies that had emerged per vial after 14 days from the cessation of egg laying. I chose this 2-week observation period to capture the maximum number of eclosed adults. Following this census, I also counted the pupal cases in each vial.

2.3 Results

Treatment with QMP significantly affected survivorship of females (Wald statistic = 24.6, d.f. = 4, $P < 0.001$; Table 2.1), where females exposed to the highest concentration of QMP were ca. 7 times ($1/\exp \beta$) more likely to die within the 48-h-treatment period than unexposed controls ($\exp \beta = 0.141$, $P < 0.001$).

All count data (i.e., egg number, number of pupae, number of eclosed adults) are normally distributed (Kologorov-Smirnov test statistics < 0.15 , with $P > 0.05$ in all cases) and are therefore analyzed without transformation. Pheromone treatment significantly affected egg number in a dose-dependent manner ($F_{4,47} = 9.87$, $P < 0.001$; Figure 2.1A–F). Concentrations of 13 and 26.5 qe resulted in the most significant reductions (mean = 6.30, $P < 0.001$, $n = 10$; 6.20, $P < 0.001$, $n = 5$, respectively), relative to unexposed controls (mean = 15.30, $n = 10$). By comparison, females exposed to lower concentrations of 3 and 6.5 qe showed only modest reductions in egg number (mean = 11.40, $P = 0.13$, $n = 10$; and 9.66, $P = 0.006$, $n = 12$, respectively), compared to controls.

Pheromone treatment significantly affected ovary area ($F_{4,45} = 11.347$, $P < 0.001$; Figure 2.1G) with higher doses corresponding to the smallest ovaries. Again, the two

concentrations of highest effect were 13 and 26.5 qe, reducing ovary area ca. two-fold, relative to unexposed controls (mean = $0.98 \times 10^6 \mu\text{m}^2$, $P < 0.001$, $n = 10$; and $0.92 \times 10^6 \mu\text{m}^2$, $P < 0.001$, $n = 5$, respectively). By comparison, females exposed to only

Table 2.1 Effect of queen pheromone (QMP) on female survivorship. Exp β denotes the change in the odds ratio as indicated by the logistic regression. Only the highest concentration of QMP (160 qe) reduced survivorship.

	QMP [qe]				
	0	3	6.5	13	26.5
Died	0	0	0	3	25
Survived	30 (100%)	35 (100%)	40 (100%)	32 (91.4%)	20 (44.4%)
Total	30	35	40	35	45
Exp β		1.29	1.589	0.908	0.141
		($P = 0.61$)	($P = 0.35$)	($P = 0.85$)	($P < 0.001$)

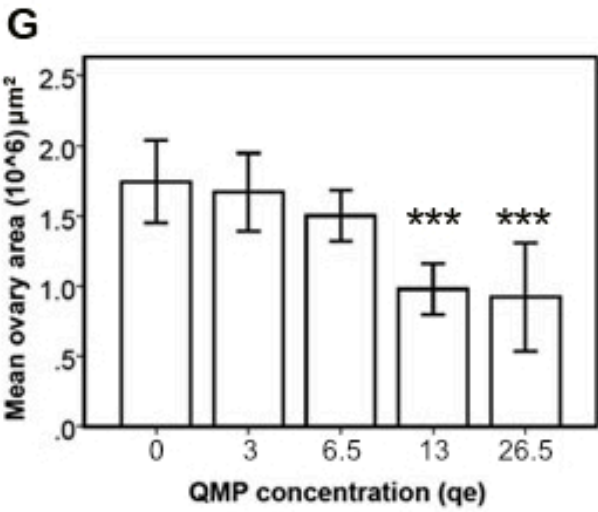
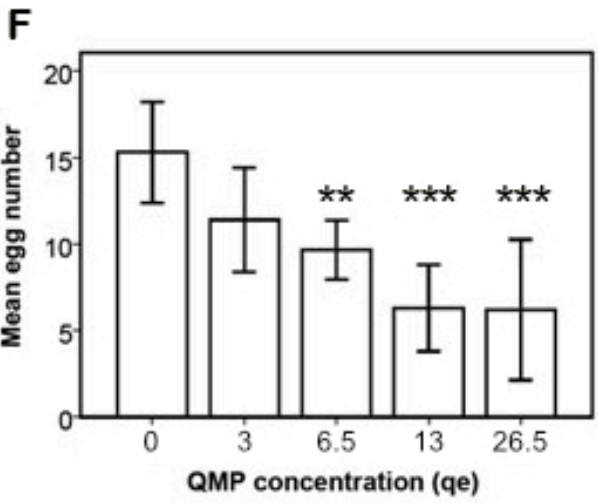
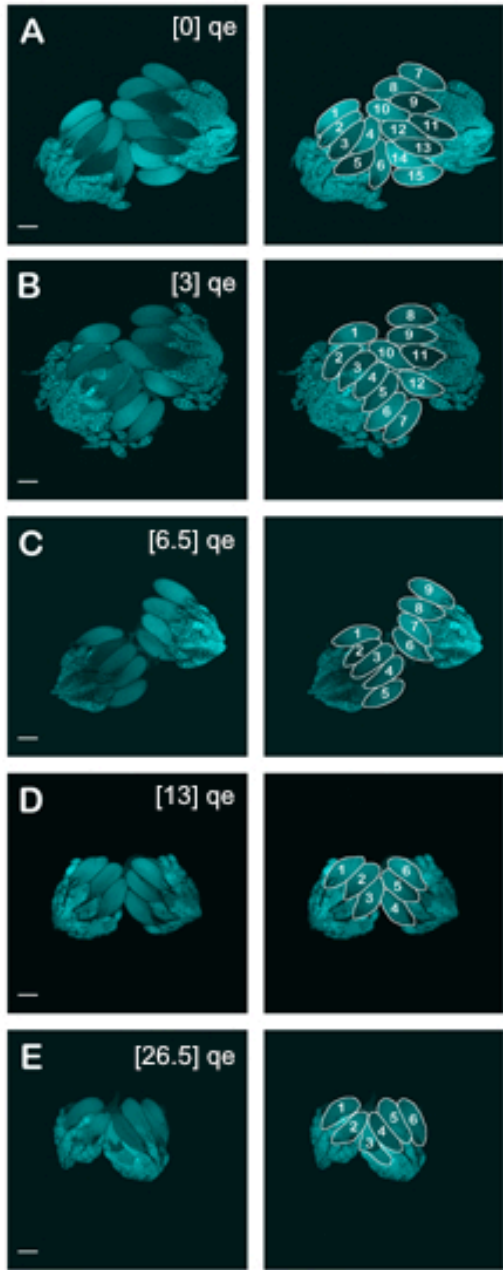


Figure 2.1 Effect of pheromonal treatment on fly ovary size and egg number. (A-E) Ovaries stained with nuclear marker DAPI after 48 hours of exposure to QMP. Rows represent different concentrations of QMP in queen equivalents and columns show original confocal z-stack projections of ovary pairs with and without tracings of mature (stage 14; King, 1970) eggs. (F-G) Relationship between QMP concentration and egg number (top graph) and ovary area (bottom graph). By either measure, treatment with pheromone inhibited ovary development. Error bars represent 95% confidence intervals and scale bars represent 200 μm . The asterices indicate statistical significance (***, $P < 0.001$; **, $P < 0.01$) compared to the [0] qe control group.

3 qe (mean = $1.67 \times 10^6 \mu\text{m}^2$, $P = 0.96$; $n = 9$) or 6.5 qe ($1.50 \times 10^6 \mu\text{m}^2$, $P = 0.33$, $n = 12$) were not different in ovary size compared to controls (mean = $1.74 \times 10^6 \mu\text{m}^2$, $n = 10$).

Pheromone treatment significantly affected the number of pupae ($F_{3,63} = 4.28$, $P = 0.008$) and number of eclosed adults ($F_{3,63} = 3.83$, $P = 0.014$; Figure 2.2) that were reared from females exposed to QMP. Specifically, females exposed to 6.5 qe produced significantly fewer pupae (mean = 3.90, $P = 0.007$; $n = 18$) and eclosed adults (3.33, $P = 0.017$; $n = 18$), compared to controls. Concentrations of 3 and 13 qe, by contrast, did not significantly affect the number of pupae ($P = 0.56$ and 0.08 , respectively) or eclosed adults ($P = 0.64$ and 0.14 , respectively), compared to controls.

To test whether the observed difference in number of eclosed adults across treatments was simply due to a failure of adults to emerge from their pupal shells following otherwise successful egg laying, I compared the ratio of pupae to empty pupal casings post eclosion. I found no difference in the number of pupae within rearing chambers to the number of eclosed adults for any of the four treatment groups ($F_{3,63} = 0.96$, $P = 0.41$). Finally, treatment with my pharmacological control pheromone 7-tricosene did not significantly affect egg number ($F_{2,33} = 0.762$, $P = 0.48$) or ovary area ($F_{2,34} = 2.60$, $P = 0.089$), relative to a hexane-only control.

2.4 Discussion

The comparative exposure of virgin female flies to honey bee queen mandibular pheromone clearly shows a cross-species effect. Specifically I show that through exposure to an ovary-regulating pheromone derived from a social insect, virgin female flies had fewer mature eggs in their ovaries, and had smaller ovaries than did unexposed controls (Figure 2.1). This pattern suggests that fruit flies are responsive to a social pheromone cue, despite not being social and thus having no selective history to suppress personal reproduction in the presence of reproducing relatives. Moreover, I found that exposure to QMP is associated with fewer pupae and fewer mature offspring (Figure 2.2). This suggests that QMP inhibits the direct reproductive potential of female flies in a

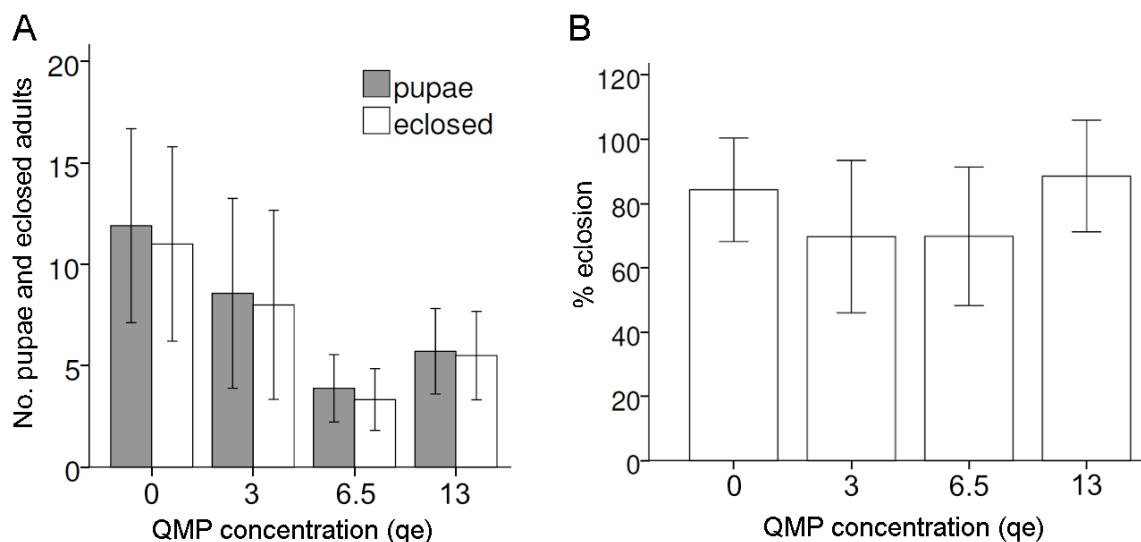


Figure 2.2 Fecundity scores of females treated with QMP. A) Counts of pupae and offspring produced from females treated with QMP. Bars represent the mean number of pupae and eclosed adults after 14 days from cessation of egg laying. B) The percentage offspring that successfully emerged from pupae from females treated with QMP. There was no significant difference in percent emergence suggesting that the difference in number of eclosed offspring among females treated with QMP was not simply due to a failure to pupate. Bars represent the mean percentage of offspring that eclosed from their pupal shells, counted after 14 days from egg laying. Data for eclosion percentage were normalized (log transformed) and added into the multivariate analysis performed above. Error bars represent 95% confidence intervals. The asterices indicate statistical significance (**, $P < 0.01$; *, $P < 0.05$) compared to the respective [0] qe control group.

manner similar to its effect on worker honey bees. This response by flies is unlikely to be adaptive given that they would not normally be exposed to a bee pheromone. Yet, the magnitude (large effect size) and direction (reproductive suppression) of the response suggests a similar physiological effect in the virgin fly and the worker bee. Moreover, the lack of effect on ovaries, even at high dose, of the alternative pheromone 7-tricosene suggests that the observed effect from QMP was not simply a pharmacological side-effect. Though I cannot strictly rule out an incidental effect, my result is consistent with a pathway-specific effect from QMP. Findings from this study are therefore consistent with the notion that social and solitary insects share regulatory networks that govern personal reproduction (Amdam et al. 2006; Bloch & Grozinger 2011; Fischman et al. 2011; Kamakura 2011).

I found that exposing fruit fly females to high concentrations of QMP resulted in a significant decrease in the number of mature eggs present in the ovaries (Figure 2.1A–F). This result is consistent with the response from *A. mellifera*, where exposure to either synthetic (Hoover et al. 2003) or queen-derived (Maisonnasse et al. 2010) QMP induces workers to suppress their ovaries (reviewed in Backx et al. 2012). My results also showed a strong dose response in which the lightest doses did not reduce egg number, but exposure above the minimal doses did reduce egg number, and in a manner roughly proportional to dose (Figure 2.1F).

Female fruit flies exposed to QMP showed a significant decrease in ovary surface area (Figure 2.1G). This effect was also shown to be dose dependent, with a pattern that closely resembled the effect on egg number (above). Egg number and ovary area are correlated traits within the Diptera (Berrigan 1991), and my finding is consistent with this general pattern. Sannasi (1969) reported a similar finding; female flies injected with a single component of QMP had ovaries of shorter length and width. Here, I used a multi-component formulation of QMP, which may be more biologically realistic given the synergistic effects that arise from interaction among individual components (Keeling et al. 2003). Moreover, my delivery of QMP to flies via a food medium, as opposed to injection, is similar to the oral delivery within bee hives via trophallaxis.

I exposed flies to different concentrations of QMP to gauge their ovarian response threshold. The two highest doses yielded similar responses, suggesting a plateau above which no further effect on reproductive physiology is observed. Furthermore, I found that the highest dose of QMP used in my study (26.5 qe) had a negative impact on survivorship (Table 2.1), suggesting that exposure to a conspicuously high dose can be toxic to flies. It is not known whether similarly high doses of QMP would be toxic even to bees, though at least one study shows that workers will avoid QMP at doses higher than normal for free-living hives (Moritz et al. 2002).

I also found that exposure to moderate (6.5 qe) and high doses (13 qe) of QMP are sufficient to impact the number of progeny a female can produce after mating, as indicated by pupal counts (Figure 2.2A) and number of adult offspring. In normal queenright colonies, worker honey bees exposed to sufficient doses of QMP are functionally sterile, and with rare exception refrain from laying (unfertilized) eggs (Visscher 1989). Although fruit flies exposed to QMP in this study did not appear to be fully sterile, they do seem to experience a reduction in fecundity. My data suggest a positive correlation between egg number and fecundity in that females exposed to QMP produced fewer eggs and produced fewer offspring.

This study suggests that flies respond to a social pheromone in a manner homologous to the honeybee. This scenario seems possible when considering comparable findings that show the potential for convergence in reproductive physiology between the genera *Drosophila* and *Apis*. For example, quantitative trait loci for ovary size in *Apis* contain genes that are homologous to those involved in ovary development in *Drosophila* (Linksvayer et al. 2009; Rueppell et al. 2011). Furthermore, genes such as *thread* (Rodriguez et al. 2002), *loki* (Oishi et al. 1998) and *quail* (Matova et al. 1999) appear to play vital roles in ovarian development in both *Apis* and *Drosophila*. Single gene studies have also revealed similarities in gene function between these two social and non-social genera. Jarosch et al. (2011) reported that alternative splice variants of a transcription factor (*gemini*) controlling oocyte development in *Drosophila* also regulates egg laying in worker honey bees. Finally, Kamakura (2011) found that consumption of royalactin induces queen-like characteristics in *Drosophila*, including increased fecundity by acting

on the EGFR signaling pathway. Together these comparisons suggest that the mechanisms governing reproductive regulation in these two taxa may be conserved.

I do not know the precise mechanism by which bee QMP might inhibit fly ovaries, but one possibility is via disruption of dopamine signaling. Dopamine is important to ovary development in *Drosophila* (Neckameyer 1996) and in worker bees (Harris & Woodring 1995). QMP may disrupt dopamine signaling to inactivate bee ovaries (Beggs et al. 2007). Given that the homovanillyl alcohol (HVA) component of queen pheromone is structurally similar to dopamine (Vergoz et al. 2009), HVA may be key to working out this mechanism and provides one specific candidate for further study. Beyond *Apis* and *Drosophila*, there is a growing body of comparative data that suggests social signals may be effective at eliciting cross-species effects. For example, colonies of drywood termites (Isoptera) exposed to honey bee (Hymenoptera) QMP produced fewer reproductives within colonies (Hrdý et al. 1959). Conversely, honey bees exposed to termite or ant queen pheromone reared fewer queens (Butler 1966).

Although my correlation between fecundity and dose does not directly test for genetic effects, the response from flies – however, measured (Figures 2.1 and 2.2) – suggests the presence of a common genetic toolkit that is conserved at the molecular level across diverse taxa (Toth & Robinson 2007). This ‘toolkit’ or ‘groundplan’ hypothesis predicts a conserved set of genes that regulate reproduction – and in particular, ovary activation – from solitary to social taxa (Amdam et al. 2006; West-Eberhard 1989). Although these genes and their pathways are only becoming known (Cardoen et al. 2011; Grozinger et al. 2007; Thompson et al. 2008), the assays presented in this study suggest that their function may be conserved from initial detection of social signal to downstream behavioural output. Future studies that test this hypothesis more generally across a wide range of taxa will help uncover the as yet unknown pathway(s) that constitute a potentially universal pathway for reproduction in insects.

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Chapter 3

3 How flies respond to honey bee pheromone: the role of the foraging gene on reproductive response to queen mandibular pheromone

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3.1 Introduction

Eusocial insects—such as ants, bees, and wasps—are characterized by a division of labour into reproductive and non-reproductive specialists. This division is particularly pronounced in highly eusocial insects like the honey bee *Apis mellifera* (Hymenoptera), whereby task specialists have evolved into morphological castes (Michener 1974).

Though the queen and worker castes differ with respect to a number of traits related to behaviour and physiology, the essential difference in reproductive potential among females rests with ovary activation and egg laying (Backx et al., 2012). The queen's ovaries contain on average several hundred ovarioles (Jackson et al. 2011), and she is capable of laying thousands of eggs per day. Workers, by contrast, have small ovaries with far fewer ovarioles (~10) that are not normally activated in the presence of the queen (Visscher 1989). As a consequence, the honeybee worker caste is considered functionally sterile.

Worker sterility is an example of reproductive altruism, whereby workers forgo their own reproductive output in order to augment the output of relatives. While inclusive fitness theory is sufficient to explain why this type of behaviour evolves (Bourke 2011), it does not in itself explain how it evolved in terms of the genes or pathways involved (Bloch & Grozinger 2011; Thompson et al. 2008). The reproductive ground plan hypothesis (hereafter RGPH) offers one explanation; it suggests that caste-based divisions in

reproductive labour ultimately evolved from the reproductive and non-reproductive phases of a solitary female's life cycle. Under disruptive selection, the alternate phases have become de-coupled to form distinct castes (Amdam et al. 2006; West-Eberhard 1996). Among extant eusocial taxa, the queen represents the reproductive phase and the worker represents the non-reproductive phase. This idea has since been extended to account for the evolution of subcastes, with one example being the 'nurse' and 'forager' workers of honey bees (Amdam et al. 2006). Even here, the idea is the same; that ancient gene sets have been subtly modified to coordinate social divisions in labour (Johnson & Linksvayer 2010).

One prediction from this solitary-to-eusocial hypothesis is that the underlying gene networks that regulate reproduction will to some extent be homologous between social and non-social taxa (Amdam et al. 2006; Page & Amdam 2007; Page et al. 2009; Toth & Robinson 2007). That is, the ovaries of eusocial taxa—despite functioning within a radically different breeding system—are regulated by similar sets of genes, as are the ovaries of solitary insects. The prospect of finding conserved genes that regulate reproduction is heightened by sociogenomic studies that routinely identify genes functionally conserved between social and non-social taxa (Fitzpatrick et al. 2005), and by experimental studies that can induce 'queen-like' qualities from non-social insects (Kamakura 2011). It is unknown, however, how conserved the whole mechanism is that regulates female reproduction in insects, social or not. If, for example, solitary fruit flies and eusocial honeybees share a common ovary-regulating pathway, then it is possible that *Drosophila* females can likewise be manipulated into de-activating their ovaries in response to social cues that normally function to induce sterility in worker honey bees.

A principal pheromone that induces worker sterility via ovary inactivation is queen mandibular pheromone (QMP). QMP is composed of five components, including carboxylic acids and aromatic compounds (Slessor et al. 1988). Kin theory suggests that queens produce QMP as an honest signal of their fecundity, to which workers are indirectly selected to attend by de-activating their ovaries (Keller & Nonacs 1993; Kocher et al. 2009). This altruistic response can depend on the age or role of the worker. For example, young nurse workers have greater reproductive potential than do older

forager workers, and it is, therefore, predictable that nurses are more responsive to QMP than are foragers (Fussnecker et al. 2011). Likewise, any response from *Drosophila* to QMP may depend on female reproductive potential.

The sitter strain of flies (*for*^S) is grossly similar to nurse bees in that they forage at shorter distances, and have lower expression of the *for* transcript (Osborne 1997). The rover strain of flies, by contrast, is similar to foraging bees in that they forage at longer distances and have higher expression of the *for* transcript. I reason that if there remains a common toolkit for reproductive regulation between social and non-social taxa, then flies may generally respond to QMP, albeit non-adaptively given that they are not social (Camiletti et al. 2013). Moreover, given the similarity between sitter/rover flies and nurse/forager bees in particular, there is the possibility for a differential response, with sitters being generally more responsive to QMP than are rovers, as are nurse bees over foragers.

In this study, I test for evidence of an underlying toolkit that regulates female reproduction across social and non-social orders. In a preliminary study, I have shown that *Oregon R* flies respond to QMP as worker bees typically do—in this case, by reducing reproductive potential through fewer eggs and smaller ovaries (Camiletti et al., 2013). Here, I build on this comparative result to test whether rovers (*for*^R) and sitters (*for*^S) show a differential response to QMP. Specifically, I compare the ovarian response of the *for*^S strain, which is characterized by low expression of cGMP-dependent protein kinase (PKG) at the *foraging* locus (Osborne, 1997), and the *for*^R strain, which is characterized by correspondingly high expression of PKG. If sitter/rover flies are differentially responsive to QMP as are nurse/forager bees, it would suggest a more striking degree of functional homology between these unrelated insect species than has previously been thought. Moreover, it would suggest a specific role for the *foraging* gene in the regulation of personal reproduction.

3.2 Methods

3.2.1 Fly rearing

I maintained *for*^R and *for*^S strains (Pereira & Sokolowski, 1993) of *Drosophila melanogaster* at 25 °C (12 h:12 h light/dark cycle) on a standard medium of yeast (*Saccharomyces cerevisiae*, Type II; Sigma-Aldrich Life Sciences, St. Louis, MO) sugar, agar (EMD Millipore, Darmstadt, Germany), cornmeal (Unico, Concord, Canada) and 0.4 % methyl-*p*-hydroxybenzoate (MP Biomedicals, Santa Ana, CA), as described in Camiletti et al. (2013). In addition, I maintained a third strain of fly—a mutant sitter strain *for*^{S2} in which a sitter mutant allele was generated on a rover genetic background (de Belle et al., 1989). I include this third strain to test for genetic background effects and the potential for inter-locus interactions (epistasis) on ovary phenotype. From homogenous stocks of each strain, I transferred groups of day- old $n = 30$ larvae into fresh food vials, then maintained each group as above until adult emergence, approximately 10 days following transfer.

3.2.2 Treatment with queen pheromone

Within 1 h of adult emergence, I subsampled groups of $n = 5$ virgin females (three groups per condition) from each strain and exposed them to one of four pheromonal treatments. The treatments consisted of [3], [6.5], [10], and [13] ‘queen equivalent’ (qe; Pankiw et al., 1996) concentrations of synthetic QMP (Contech Enterprises Inc. Canada, <http://contech-inc.com/products/apiculture/>), as estimated by weight from an ethanol-diluted stock solution (500 mg \approx 1,184 qe). I also included a control treatment that consisted of just ethanol (no pheromone). I kept all treatment solutions at –20 °C, and warmed them in a 50 °C water bath prior to use.

I exposed groups of $n = 5$ females to one of the five treatments within custom-made chambers. Chambers consisted of a 50 ml Falcon tube with the funnel tip melted off and replaced with a cotton plug. I then fit the Falcon tube cap with a circular piece of filter paper (grade 413, VWR International LLC, Radnar PA) that we saturated in yeast and sugar solution (0.1 g yeast, 0.15 g sugar, in 5 ml of 5 % ethanol). I pipetted a volume of 20 μ l of each treatment solution onto the center of the filter paper before re-fitting the

cap. For the control treatment, I simply used 20 μ l of 100 % ethanol, containing no QMP. I exposed each group of flies within these custom chambers for a period of 48 h, after which I CO₂ narcotized all flies, and dissected their ovaries under an Olympus S7X7 stereomicroscope, fitted with an Olympus SZ2-LGD1 light source (Olympus, Richmond Hill, Canada).

3.2.3 Ovary assay

I dissected left and right ovaries using forceps and a medium of 1 \times Dulbecco's phosphate-buffered saline (1 \times D-PBS; Invitrogen, Carlsband, CA). I then fixed each set of ovaries within 1 h of dissection (4 % formaldehyde in D-PBS for 20 min), then washed (D-PBS three times over 15 min), washed again [1 \times PBT (0.001 % Triton X in D-PBS) for three times over 15 min. and, finally, stained ovary tissue with DAPI (1:2,000) in D-PBS for 1 h on a shaking plate. I mounted all stained ovaries in 7 % glycerol in D-PBS prior to visualization under a confocal microscope (Zeiss LSM 510). For each female, I used the confocal microscope to count the total number of mature eggs (stage 14; King, 1970) for each pair of ovaries. I used image acquisition software (ZEN 2009 Light Edition; Carl Zeiss Canada Ltd., Toronto, Canada), and the 'count size' macro of the software Image Pro Plus 7.0 (Media Cybernetics, Bethesda, MD), to estimate the area (square micrometer) of each ovary as a function of strain ($n = 3$) and treatment group ($n = 5$). Ovaries where light intensity contrasts were insufficiently sharp to permit an accurate estimate of area were discarded (11 % of total used for egg counts). I used univariate ANOVAs to evaluate the effect of strain and treatment on ovary area, and on egg number. For statistical analysis I used IBM Statistics SPSS 19.

3.3 Results

I dissected and scored the ovaries (left and right) from a grand total of 165 flies reared under different pheromone treatments (Table 3.1). I found a significant main effect of genetic strain on the number of eggs within ovaries ($F_{2,150} = 203.03$, $P < 0.001$) and on ovary area ($F_{2,141} = 52.54$, $P < 0.005$). Of note is the substantial difference among the three strains for either character even in the absence of QMP; the *for*^S strain showed the

highest egg counts and largest ovary areas. The *for*^{S2} strain, by contrast, consistently showed the lowest egg numbers and smallest ovary areas (Fig. 3.1).

I also found a significant effect of pheromone treatment on the number of eggs within ovaries ($F_{4,150} = 11.12$, $P < 0.005$) and on ovary area ($F_{4,141} = 6.74$, $P = 0.012$). The *for*^S strain had the most predictable response to QMP; females in this treatment group showed declining egg counts and ever smaller ovary areas in response to increasing doses of pheromone (Fig. 3.1). The *for*^R strain, by contrast, showed an initial increase in egg number and ovary area upon exposure to low levels of QMP, followed by a return to baseline numbers of eggs and ovary size at the highest concentrations. The *for*^{S2} strain did not show a large or consistent response to QMP. This strain showed consistently low egg counts and small ovary areas across all concentrations, relative to the other two strains (Fig. 3.1).

Finally, there was a significant pheromone \times strain interaction effect whereby the effect of dose depends on the fly's genotype for both egg number ($F = 6.33$, $P < 0.001$) and ovary area ($F = 5.02$, $P < 0.005$). Sitters are increasingly responsive to higher doses of pheromone; whereas, rovers show a more complex pattern for both measured traits and appear to be inhibited only at the highest doses (Fig. 3.1 a, b).

3.4 Discussion

In this study, I show that young female fruit flies are apparently responsive to an interspecific pheromonal cue. In general, sitter flies exposed to doses of honey bee QMP developed fewer eggs and smaller ovaries than did untreated controls (Fig. 3.1, panel a); however, the response from rover flies was less predictable, suggesting a strong gene \times environment interaction effect on measures of personal fitness (Fig. 3.1, panels b, c). Taken together, my results highlight the potential for an interspecific effect from social cues, even on—in this case, an unrelated non-social taxon. My results are, therefore, consistent with the hypothesis that social and non-social species can remain functionally conserved with respect to mechanisms that regulate reproduction. If so, this mechanism may involve the *foraging* gene.

Table 3.1 Number of female flies used for egg count and ovary area measurements for each strain (for^s , for^R , and for^{s2}) and at each QMP dose (qe; [0], [3], [6.5], [10], and [13]). Within each strain and QMP dose, the same flies were used for both egg count and ovary area measurements. Ovaries not suitable for area measurement were only included in egg count analysis.

	for^s		for^R		for^{s2}	
QMP dose (qe)	Egg count	Ovary area	Egg count	Ovary area	Egg count	Ovary area
[0]	11	9	11	11	9	9
[3]	10	11	10	10	11	11
[6.5]	14	12	8	8	13	12
[10]	12	11	10	10	10	10
[13]	13	12	10	10	13	10
TOTAL	60	55	49	49	56	52

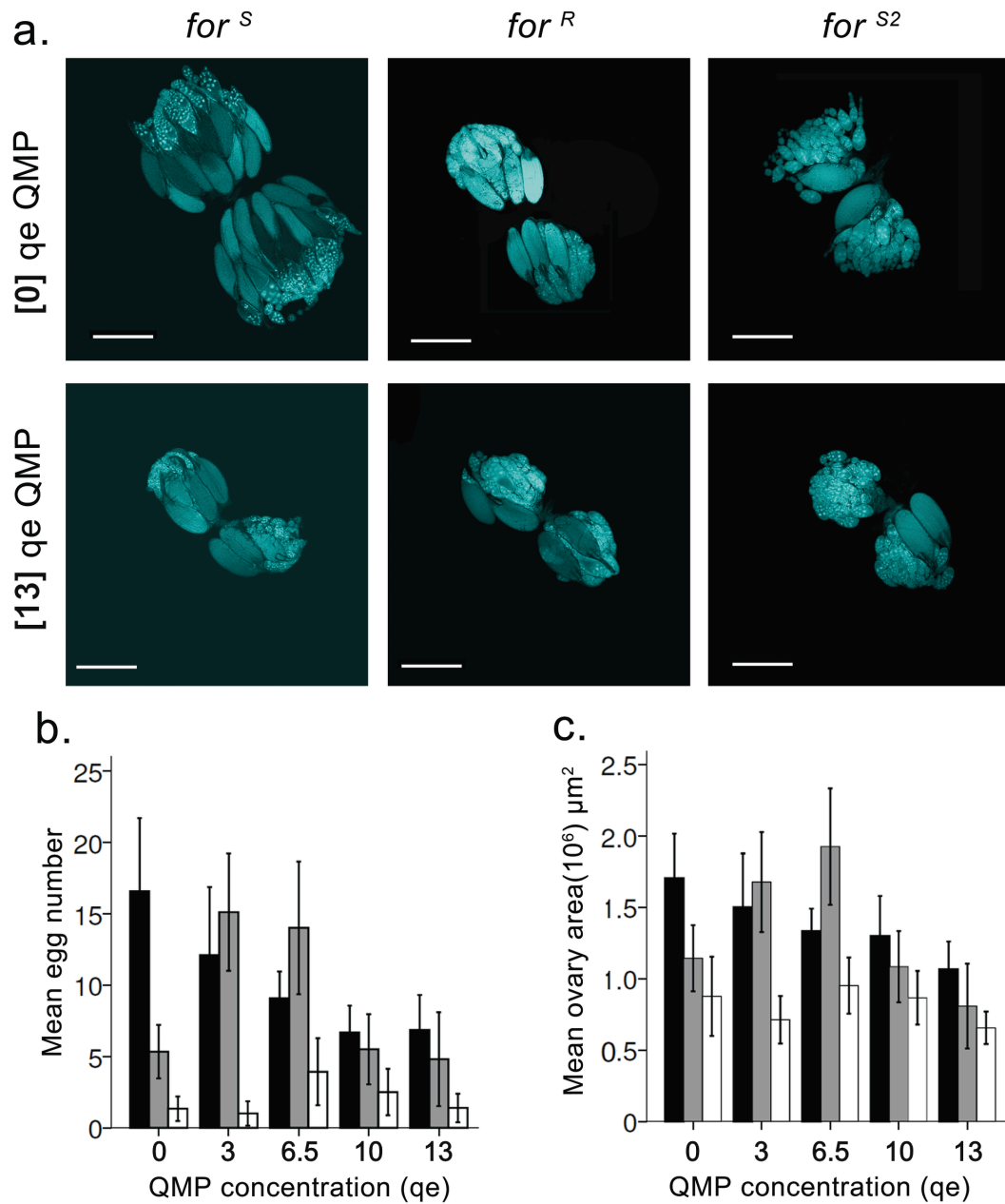


Figure 3.1 Effect of pheromone treatment and fly strain (*for^S*, *for^R*, and *for^{S2}*) on ovary egg number and ovary area. (a) Images show representative pairs of ovaries stained with nuclear marker DAPI after treatment with no QMP (top row) and after treatment with [13] qe of QMP (bottom row). Columns represent the different fly strains as indicated. Scale bars represent 500 μm . Graphs of mean egg number (b) and mean ovary area (c) for *for^S* (black bars), *for^R* (gray bars), and *for^{S2}* (white bars). Error bars represent 95% confidence intervals.

The potential for interspecific effects of honey bee-derived social cues on the reproductive phenotype of fruit flies is a recent topic of study. First, Camiletti et al. (2013) showed that wild type *Oregon R* virgin females exposed to honey bee queen mandibular pheromone yielded fewer eggs and developed smaller ovaries than did untreated controls. Moreover, the *Oregon R* flies used in that study appeared to respond to QMP in a dose-dependent manner, whereby higher doses of pheromone further inhibited the personal fitness of egg-laying flies. Though Camiletti et al. (2013) do not strictly rule out a pharmacological effect, the direction (inhibition) and magnitude (dose dependence) of the effect is comparable to the normal (and adaptive; Crozier & Pamilo, 1996) effect of QMP on honey bee workers. Second, in a comparable study, Kamakura (2011) demonstrated that female fruit flies are developmentally responsive to another honeybee-derived cue—namely, royalactin, an inducing factor in royal jelly that, when fed to developing bee larvae, can bias their reproductive fate (Drapeau et al. 2006; Haydak 1970). Kamakura (2011) showed that female *Canton-S* flies fed royalactin had larger bodies, laid more eggs per day, and lived longer compared to flies reared on standard diets. This conspicuous ‘queen-like’ response suggests that not only can *Canton-S* flies respond to this honey bee-derived nutritional cue to which they would never normally be exposed, but do so in a manner that is analogous, if not homologous, to the typical response from honey bees themselves. These studies keep open the idea that genetic pathways regulating reproductive division of labour are noticeably conserved between solitary to eusocial species (Page et al. 2009; Smith et al. 2008; Toth & Robinson 2007).

The present study builds on the results of Camiletti et al. (2013) and Kamakura (2011) to elucidate the role of genotype in mediating the fly’s response. I employ the well-known sitter and rover fly strains that differ in gene content at the *foraging* locus (Osborne 1997), and which are loosely analogous to the specialized nurse and forager worker subcastes of honey bees (Ben-Shahar et al. 2002). Since nurse bees are young and perform in-hive tasks related to brood care, they presumably have greater reproductive potential and greater egg-laying opportunities than do forager bees. Under selection, QMP should therefore elicit a stronger effect from nurse bees (Fussnecker et al. 2011) or, by analogy, sitter flies. I show that sitters do score higher on both measures of personal

fitness than do rovers (Fig. 1, panel b). That is, in the absence of any pheromone, female flies carrying the sitter allele at the *foraging* locus (*for*^S) have ~2.4 times as many eggs in their ovaries as do rovers (Fig. 3.1, panel b). Likewise, *for*^S females have ~1.5 times larger ovaries than *for*^R females (Fig. 3.1, panel c). Given that these strains are characterized by differences in allelic composition at the *foraging* locus, this pattern suggests a genetic effect on ovary phenotype whereby egg number and ovary size are positively associated with specific *foraging* alleles. The precise mechanism through which *foraging* acts on ovaries is not known but one possibility is via the tumor suppressor gene PTEN (*phosphatase* and *tensin*) that is functionally associated with differential ovary activation between nurse and forager bees (Mutti et al. 2011). It should also be possible to test if the response from *Drosophila* to QMP is mediated through olfactory pathways—for example, by measuring the response in *Or83b* (or similar) flies that are mutant for a critical olfactory co-receptor proteins (Larsson et al. 2004).

A secondary genetic effect on ovary phenotype is revealed by the *for*^{S2} strain. In the absence of any pheromone, females of this genotype have the fewest eggs and smallest ovaries of any strain, despite carrying a sitter mutant allele (Fig. 3.1, panels b, c). Given that the *for*^{S2} strain is characterized by the presence of a sitter-like allele in a rover background (de Belle et al. 1989), the diminished phenotype may reflect a new-found and inherently different effect of the sitter (*for*^S) versus sitter mutant (*for*^{S2}) allele on ovary phenotype. Alternatively, the differential response between sitter and sitter-mutants could reflect an interaction between the sitter mutant allele and the rover background. This study does not discriminate between these two mode of action hypotheses but the potential for the latter scenario is consistent with studies that reveal other forms of epistasis involving the *for* locus (Kent et al. 2009; Ruppell et al. 2004).

Finally, my results suggest a strong $G \times E$ interaction effect. That is, the effect of dose depends on the fly's genotype. Sitters (*for*^S) showed a more-or-less linear dose-dependent decrease in egg number and ovary area with increasing concentrations of QMP (Fig. 3.1, panels a, b); whereas, rovers (*for*^R) seem to increase ovary function over intermediate doses but not so at higher doses (Fig. 3.1, panels b, c). The response to QMP in flies is therefore dependent on the *foraging* genotype. Although the characteristic rover

and sitter phenotypes are largely defined by their genetic differences at this single locus (Osborne 1997), specific behaviours within the rover-sitter syndrome, including developmental resilience to stress (Vijendravarma et al. 2012), ability to acquire food (Kaun et al. 2007) and locomotion (Riedl et al. 2005) all show genotype-specific reaction norms (Sokolowski 2001) suggesting that alternate *foraging* genotypes are differentially sensitive to changes in the environment. The $G \times E$ interaction effect observed in the present study is also consistent with Burns et al. (2012) who further show the potential for $G \times E$ interaction effects in rover-sitter variants, and show how changes in larval feeding environment led to differences in reproduction in sitter, but not rover, flies. My study likewise showed that rovers were less sensitive to environmental manipulation.

My results do not strictly rule out a pharmacological side-effect of high-dose pheromone (of any type) on fly physiology, but the following considerations suggest a pathway specific response. First, in a comparable study, Camiletti et al. (2013) included a non-QMP pheromone control that yielded no effect on fly ovary phenotype, even at high doses. Second, the differential response observed among strains in the present study again suggests that QMP is affecting a *for*-related pathway that influences ovary activation, rather than simply being generally disruptive to flies. While ongoing experiments will help decipher general from specific effects, as well as the pathways involved, I remain curious about why sitter (present study), *Oregon R* (Camiletti et al. 2013) and other (Sannasi 1969) *Drosophila* flies appear to so closely mimic the response to QMP from worker bees themselves.

The pathway responsible for ovary signaling in the honeybee remains elusive, but it seems likely to interact with juvenile hormone (JH) and vitellogenin (Vg) as general regulators of insect reproduction (Amdam & Omholt 2003). In this study, the differential response between rovers, sitters and sitter-mutants to ovary-regulating pheromone suggests that *foraging* may also be involved in ovary signaling in the fly and potentially in the bee. The *Apis mellifera* homolog to *foraging* (*Amfor*) does appear to be functionally related to worker reproductive potential, and to JH and Vg. Nurses with low levels of *Amfor* (Ben-Shahar et al. 2002) also have higher levels of Vg in their fat bodies, and *Amfor* may be indicative of reproductive potential (Ihle et al. 2010). Further, both Vg

and JH have been shown to regulate *Amfor* expression in the fat body towards carbohydrate metabolism (Wang et al. 2012). It would be worth extending the present study to include measures of fly gene expression, including yolk protein expression, especially given the role that the yolk precursor vitellogenin plays in worker bee ovary activation (Thompson et al. 2008). In addition, it should now be possible to verify the involvement of the *foraging* gene using the GAL4-UAS targeted gene expression system that is applicable to the *Drosophila* model (Belay et al. 2007), and also to locate the tissue where PKG acts to mediate the pheromone's signal.

The results presented here show that sitters and rovers are capable of responding to QMP in a similar fashion to that seen in nurse and forager bees. This ability of fruit flies to respond to a social environmental cue suggests that solitary taxa may share conserved ovary-regulating pathways with eusocial species. Homologous gene networks between honey bees and flies have been shown for a suite of other behaviours, including those related to learning and memory (Velarde et al. 2006), and circadian cycles (Eban-Rothschild & Bloch 2012). The degree to which ovary-regulating pathways are homologous between *Drosophila* and *Apis* can now be further tested. The results presented here suggest that the *foraging* locus may be a part of a shared pathway and may generally serve to modulate the responsiveness of the ovaries to environmental stimuli.

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Chapter 4

4 RNAi-screen reveals genes for functional sterility in a female insect

4.1 Introduction

The evolution of altruism has long intrigued biologists interested in the origins of behavioural diversity (Dugatkin 2006). Reproductive altruism – of the type typical of sterile worker and defensive castes of the social insects (Wilson 1971) – is obviously costly to the selfless individual, but can, nonetheless, evolve via indirect fitness effects obtained from helping reproducing relatives (Hamilton 1964). Despite this central prediction from inclusive fitness theory, we do not yet have a good understanding of which genes are under indirect selection or that are otherwise involved in mediating the expression of altruistic behaviour (Keller 2009; Linksvayer 2015; Thompson et al. 2013).

This molecular shortcoming may stem in part from the relative youth of sociogenomics, a growing field that seeks to uncover the molecular basis of social life (Fischman et al. 2011; Johnson & Linksvayer 2010; Keller 2009; Rehan & Toth 2015; Robinson et al. 2005; Smith et al. 2008), and from current limits on the capacity for functional screening of social insect genomes (Ament et al. 2012; LeBoeuf et al. 2013). The European honey bee *Apis mellifera* is pre-eminent among post-genomic eusocial models (Denison & Raymond-Delpech 2008; Weinstock et al. 2006), and provides one opportunity to associate genome-wide polymorphisms with the evolution and expression of worker reproductive altruism (Mullen & Thompson 2015).

Honey bee researchers have generated lists of genes directly implicated in worker sterility, mostly using microarrays to screen for genes responsive to ovary-inhibiting queen pheromone (Cardoen et al. 2011; Grozinger et al. 2007; Thompson et al. 2008). These gene-level data are being assembled into multi-gene networks that provisionally describe the molecular mechanism of how an individual worker regulates her ovaries in response to queen pheromone (Chandrasekaran et al. 2011; Mullen et al. 2014). Despite this progress towards understanding how the socio-genetic environment regulates reproduction, we are still far from a top-to-bottom pathway that describes how queen pheromone triggers sterility via ovary de-activation (Backx et al. 2012).

We do know that this pathway should be attuned to queen mandibular pheromone (Bloch & Grozinger 2011), composed of five mandibular gland semiochemicals (Slessor et al. 2005). QMP is, in effect, an honest signal of fecundity to which the workers are selected to attend by de-activating their ovaries and otherwise adopting alloparental roles within the colony (Peso et al. 2015; Ratnieks & Helantera 2009). Any pathway mediating sterility is, therefore, likely to be fronted by olfactory receptors (ORs; Jarriault & Mercer 2012; Wanner et al. 2007). One approach towards orienting the pathway or otherwise determining its most up-stream components would be to screen honey bee ORs (Robertson & Wanner 2006) for functional associations with QMP, perhaps via knock-downs coupled with behavioural or ovary assays. OR-specific knock-downs remain challenging in honey bees (Jarosch & Moritz 2011; Reinhard & Claudianos 2012), but it may be useful to screen a surrogate insect for which this type of screen is immediately feasible, and that is also responsive to QMP.

The fruit fly *Drosophila melanogaster* is not social (it lacks a caste system and shows no reproductive self-sacrifice) but has proven a useful adjunct in the search for genes that underpin social traits (Ben-Shahar et al. 2003; Kamakura 2011; Rajakumar et al. 2012; Søvik et al. 2015). In one such study, Camiletti et al. (2013) showed that *Oregon R* females treated with QMP express a worker-like phenotype; they tend to have smaller ovaries that contain fewer mature eggs. This response to bee pheromone in the fly implies a degree of conservation in the genetic toolkit that regulates female reproduction between social and non-social orders. Eusocial insects have likely re-tooled this kit under selection to optimize female reproduction within a social context (Peso et al. 2015; Toth & Robinson 2007). Further, the *forager* mutant response observed by Camiletti et al. (2014) suggests that this gene in particular interacts with the pathway that regulates the female fly's response to social pheromone.

In this study I use a *Drosophila* model to screen for loci involved in the olfactory response to queen mandibular pheromone. First, I use a bioassay to quantify the extent to which the fly's response to QMP is strictly olfactory, as opposed to gustatory. Second, I screen the near-full complement of *Drosophila melanogaster* olfactory receptors via RNAi-mediated knock-downs. Individual ORs that block the fly's conspicuous worker-like response to QMP represent functional candidates for the olfactory perception of honey bee queen pheromone. Finally, to the extent that receptors identified from the fly are homologous to those from the bee, then my *Drosophila* model may for the first time help to identify genes that are firstly responsive to environmental stimuli, the immediate early genes, that are involved in the QMP-mediated worker sterility pathway. These

genes represent a poorly understood mechanism but of major significance to insect sociobiology.

4.2 Methods

4.2.1 Fly rearing

I reared all strains of *Drosophila melanogaster* under standard conditions (25°C, 60% humidity and a 12h:12h light: dark cycle) in an insect growth chamber (Caron Inc., Marietta, OH) on a standard cornmeal diet (as described in Camiletti et al. 2013). I synchronized adult emergence by first housing (for 24 hrs) a small reproductive population (n = 30 males and n = 30 females) in collection cages (60mm; Diamed, Mississauga, Canada) fitted with nutrient (grape juice and agar) plates. I then collected and transferred day-old larvae to fresh food vials (28.5 x 95mm, VWR International, Radnor, PA) at a density of n = 30 larvae per vial. Finally, I collected same-age (within 1 h of eclosion) adult virgin females, and dissected their ovaries after a further 48 hours (Figure 4.1).

4.2.2 QMP treatment

I exposed newly eclosed females to a synthetic blend of honey bee queen pheromone (Contech Ltd, Victoria, Canada). First, I diluted a 500 mg stock of QMP with 100% ethanol into two working concentrations; a dose of approximately 13 queen equivalent (qe) units (Pankiw et al. 1996) and a higher dose of approximately 20 qe units. This range of concentrations has been shown to effectively suppress fly ovaries in a manner comparable to its normal effect on worker bees (Camiletti et al. 2013). Second, I warmed working aliquots to 50°C in a water bath, and exposed flies to QMP in one of following

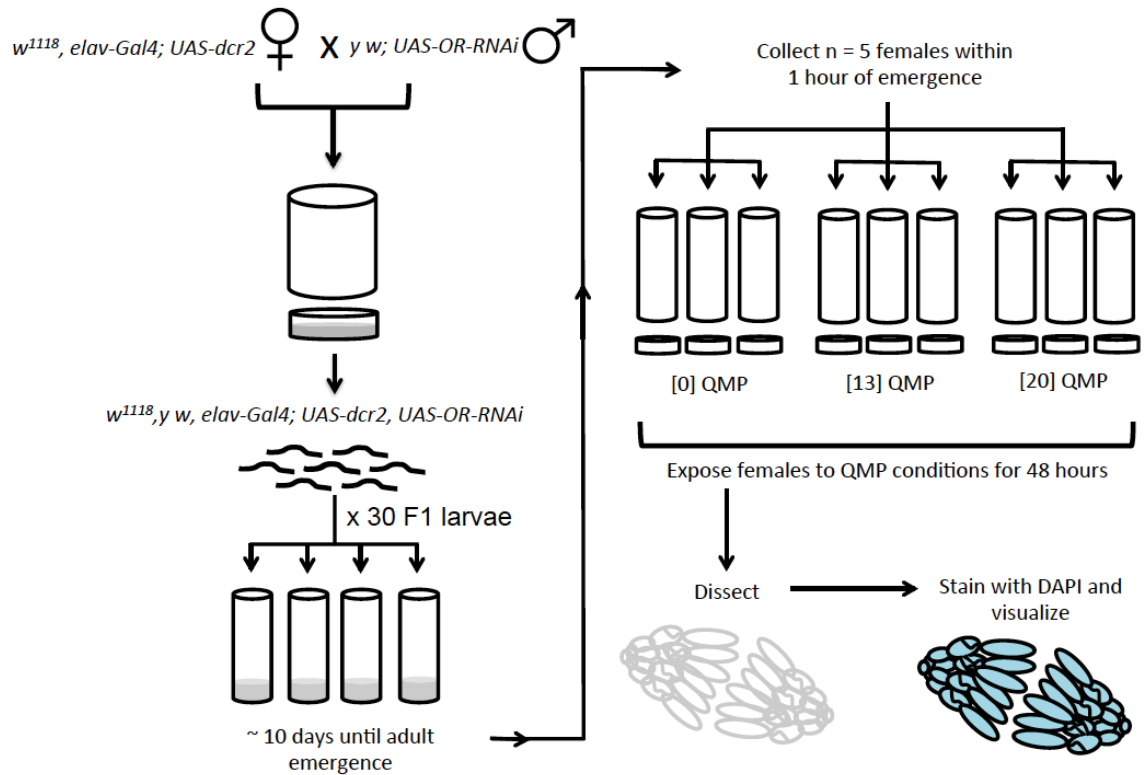


Figure 4.1 Exposure of RNAi flies to queen bee pheromone. I crossed *elav-GAL4*, *UAS-dcr2* females to males with specific *UAS-OR-RNAi* genotypes, corresponding to the $n = 48$ OR knock-downs available for *Drosophila* (VDRC Stock Center). I collected groups of $n = 30$ F1 larvae and reared them for ~10 days to maturity. I then exposed small groups ($n = 5$) of mature same-aged (within 1 hr) females to the QMP treatment. Flies received either no-QMP, or a low [13 queen-equivalents] or high [20 queen-equivalents] dose. Finally, after 48 hrs I dissected complete sets of ovaries, stained them with DAPI, and scored them against an established scale (King 1970) for assessing reproductive readiness, and did so via digitized confocal images.

two ways. Under “full access” I exposed flies to QMP within chambers that permitted full physical contact with the pheromone-treated filter paper. Under “limited access”, by contrast, I exposed flies to QMP within chambers fitted with a screen that prevented physical contact with the pheromone-treated filter paper. Perception of pheromone within this latter type of chamber is therefore presumed to be via near-distance olfaction (Figure 4.2A).

For full access trials I placed $n = 5$ flies into a 50 ml Falcon tube modified to administer pheromone (as described in Camiletti et al. 2013). Briefly, I cut the bottom tip of the tube to insert a standard fly plug, then custom fit a piece of filter paper (grade 413: VWR International, Radnor, PA) saturated with a yeast and sugar solution (0.1g yeast, 0.15g sugar, in 5ml of 5% ethanol) under the screw cap. To treat flies, I pipetted 20 μ l of QMP-EtOH solution, or the equivalent volume of just-EtOH control, onto the paper, and incubated the whole chamber for a period of 48 hrs. For limited access trials, I performed a comparable procedure, except used a mesh barrier to prevent flies from touching the filter paper. Following treatment, I dissected the ovaries of individual flies, and scored the approximate level of activation in two ways; by counting the number of mature eggs (cf. King 1970) per female (both ovaries) and by estimating the total ovary area, as inferred from on-screen measurements of digitized confocal microscope images.

4.2.3 *Orco* mutant screen for perception of pheromone

To test for genetic effects on the perception of QMP by flies, I used both full and limited access chambers to screen the ovarian response across three genotypes. Specifically, I compared the ovarian response of small groups ($n = 5$) of *Oregon R* (*Ore-R*, Bloomington Stock Center) females against two groups of mutant flies deficient for the major olfactory

co-factor *Orco* (formerly, Or83b). *Orco* is broadly expressed across olfactory sensory neurons and functionally associates with olfactory receptors to initiate neuronal signaling (Carraher et al. 2015). The mutant genotypes are: $w^{1118}; Orco^1$ and $w^{1118}; Orco^2$ (BSC ID #23129 and #23130). They are each homozygous for loss-of-function alleles (Larsson et al. 2004) – characterized by disrupted reading frames, and both are effective at blocking a wide range of olfactory stimuli (Steck et al. 2012).

4.2.4 Olfactory receptor knock-down screen for perception of pheromone

To uncover specific olfactory receptors important in the perception of QMP I took advantage of knock-down lines that individually modulate the expression of ORs via a Gal4-driven RNAi insertion (Dietzl et al. 2007; available from Vienna Drosophila RNAi Center). Here, I crossed custom-ordered *UAS-RNAi* males of either P-element RNAi ("GD Library") or phiC31 ("KK Library") genetic background with virgin $w^{1118}, elav-Gal4; UAS-dcr2$ females to generate F1s that express OR-specific knock-downs (Figure 1). I only proceeded with lines where their genetic background had a minimal effect on ovary phenotype. To select for this, I compared each knockdown line to background control F1s produced from crosses between *elav-Gal4; UAS-dcr2* females and either GD or KK males (with no QMP). From each of these pairwise comparisons, I considered only those knockdown lines for which the standardized difference in mean ovary scores was 'small' - *i.e.*, Hedge's *g* less than 0.5 (See Appendix C). For these lines only, I proceeded to measure the knock-down effect itself, again using Hedge's *g*, except in this case by simply comparing QMP-treated vs. untreated flies.

4.2.5 Scoring the level of ovary activation

For all assayed flies, I exposed females within chambers for 48 hrs. I then CO₂ anesthetized them and dissected complete pairs of ovaries from individual females using ultra-fine forceps under an Olympus S7X7 stereomicroscope (Olympus, Richmond Hill, Canada) that I fitted with a cold light source (KL300 LED; Leica Microsystems, Wetzlar, Germany). I dissected ovaries in 1X Dulbecco's phosphate-buffered saline (1x D-PBS; Invitrogen, Carlsbad, CA), then fixed stained tissue in a 4%-formaldehyde in D-PBS solution for a period of 20 min. I then washed [1 x D-PBS and 0.5% PBT (0.1% Triton X 100 in 1 x D-PBS)] and DAPI-stained (1:2000) ovaries prior to mounting (7% glycerol in D-PBS) and visualized them using a Zeiss LSM 5 Duo Vario confocal microscope (Zeiss, Oberkochen, Germany). Finally, I scored ovaries against two biological criteria that capture QMPs effect on reproductive readiness. First, I counted the number of mature (stage 14; King 1970) eggs within each ovary. I then estimated ovary area (μm^2) from confocal images using the 'thresholding' function of IMAGE-PRO PREMIER 9.1 software (Version 9.1, Media Cybernetics, Bethesda, MD). For this part of the analysis, I excluded any ovaries that were inadvertently damaged during dissection or that otherwise had weak imaging (a minority, ~3-5%).

4.2.6 Stoichiometric analysis of olfactory ligands to pheromone components

Following my screen, I compared the structural similarity of candidate olfactory receptor ligands to the five components of QMP: 9-ODA (E)-9-oxodec-2-enoic acid), HOB (methyl *p*-hydroxybenzoate), HVA (4-hydroxy-3-methoxyphenylethanol) and *cis* and *trans* 9-HDA (9-hydroxydec-2-enoic acid). First, I identified the dominant ligand for

each candidate OR using the on-line Database of Odorant Receptors (Galizia et al. 2010). I then used the maximum common sub-structure (MCS) method of Cao et al. (2008) to predict the affinity of ligands to the individual components of QMP. For each test ($n = 21$ ligand-by-component comparisons), I used the CHEMMINE application (Backman et al. 2011) that generates a Tanimoto (Rogers & Tanimoto 1960) 'similarity score' for each pair of compounds (MSC Ts). In this context, a high score (maximum of '1') implies a higher chemical identity between fly ligand and bee pheromone.

4.3 Results

4.3.1 Effect of queen pheromone on fly ovary phenotype

Oregon-R females exposed to QMP showed significantly fewer eggs ($F_{2,67} = 78.91$, $P < 0.001$) of smaller size ($F_{2,53} = 16.74$, $P < 0.001$) than did untreated controls in the full access condition. This effect of QMP on the reproductive physiology of female flies is consistent with Camiletti et al. (2013), who found that females exposed to queen pheromone had reduced ovary size, fewer mature eggs and lower realized fecundity. Flies in the limited access condition showed a similar pattern. QMP exposed females yielded fewer eggs ($F_{2,67} = 37.95$, $P < 0.001$) of smaller size ($F_{2,52} = 17.19$, $P < 0.001$) than non-exposed controls. My bioassay therefore shows that the ovarian response to QMP does not strictly require physical contact with the source of pheromone, as evidenced by the worker-like response from the limited access trails (Figure 4.2B). The *Orco* mutants, by contrast, were not affected by the pheromone. The number and size of eggs in *Orco* females did not vary upon treatment, unlike the background (w^{1118}) or wildtypes (*Ore-R*) that did contain fewer eggs ($F_{3,144} = 8.90$, $P < 0.001$) of smaller size ($F_{3,145} = 7.26$, $P < 0.001$) under QMP (Figure 3).

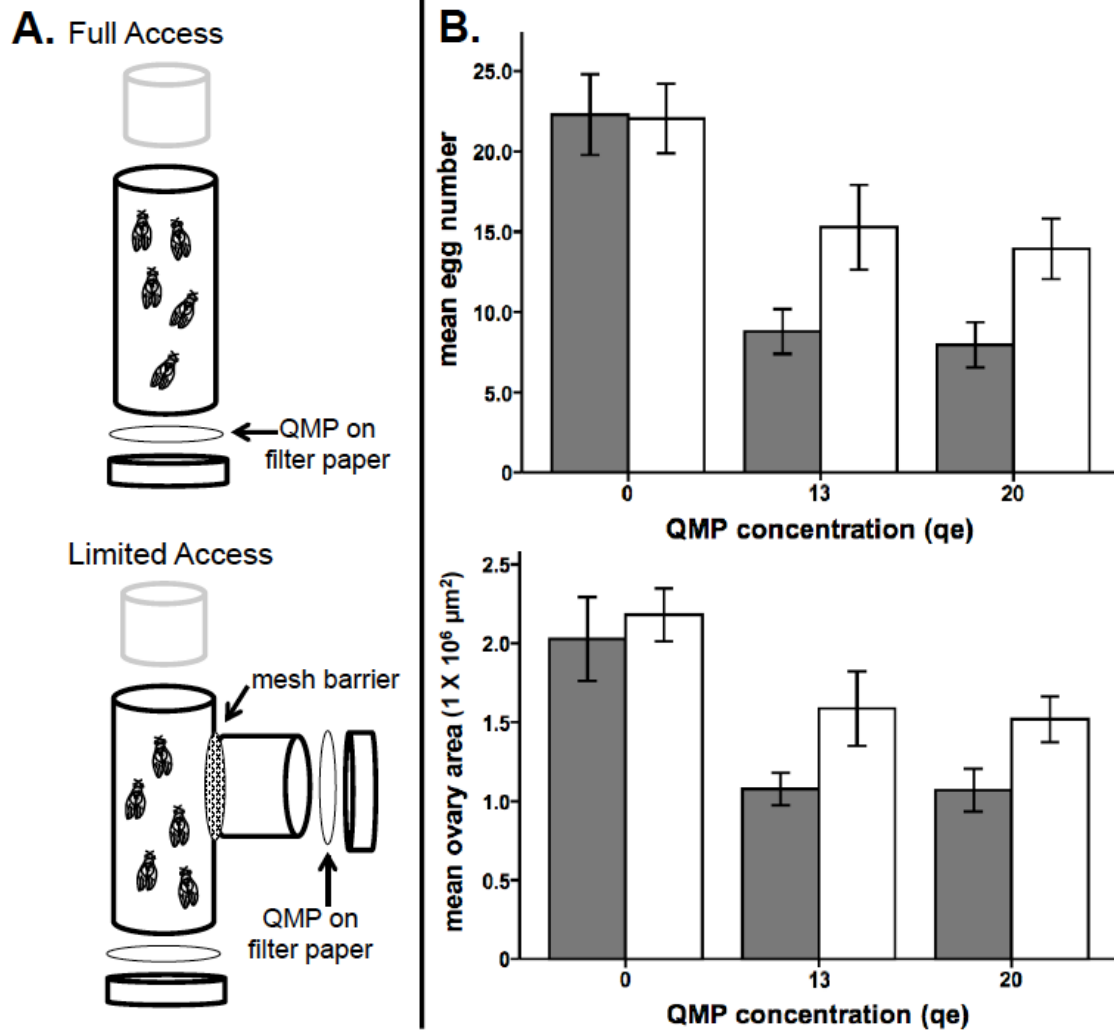


Figure 4.2 Measuring wildtype response to QMP. (A) I used 'full' or 'limited' access chambers (see text) to expose groups ($n = 5$) of wild type (*Oregon R*) flies to filter paper containing honey bee queen mandibular pheromone (QMP) or a no-QMP control. In full access chambers, flies could touch the filter paper. Under limited access, they could not. (B) Response of wild type flies to QMP treatment under full (gray bars) or limited (white bars) access. Both response variables (egg number, ovary area) decrease by 20 to 64% under QMP treatment (as in Camiletti et al. 2013), and this response is more pronounced in the full access condition. Error bars indicated 95% confidence intervals.

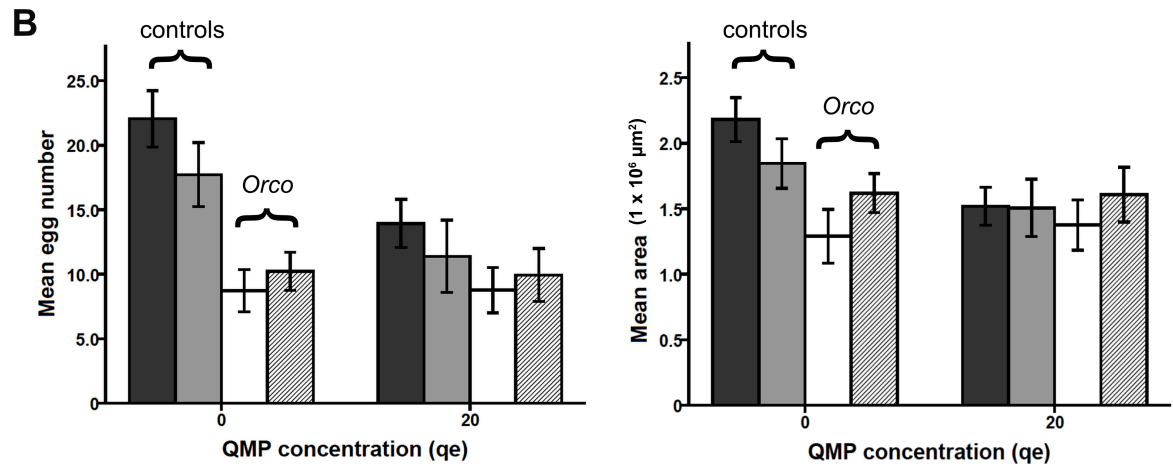
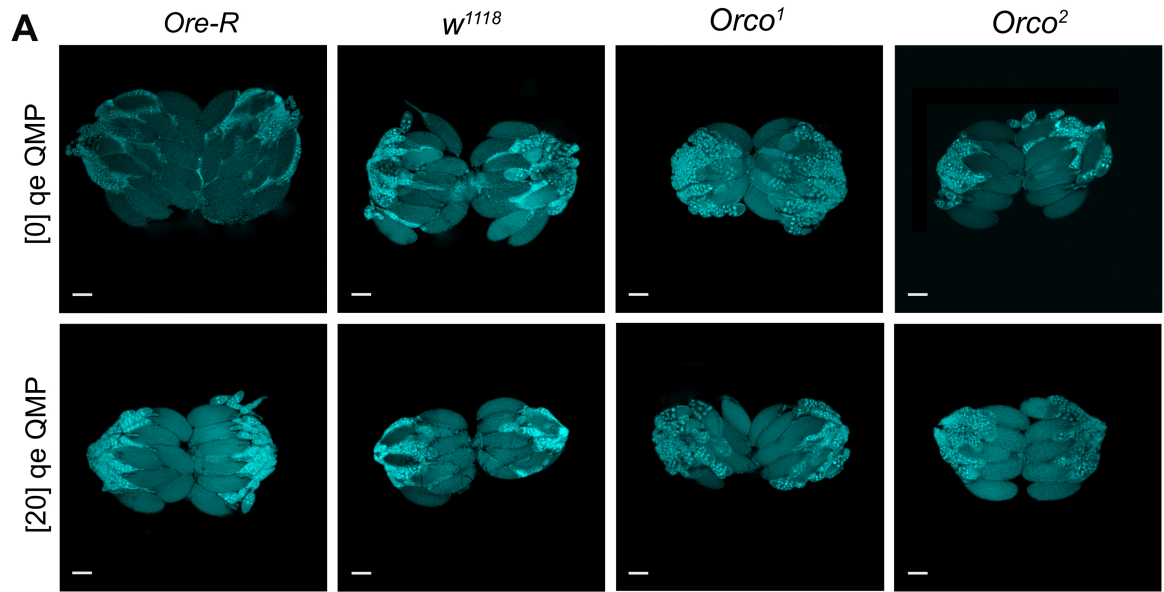


Figure 4.3 Measuring *Orco* mutant response to QMP. I used 'limited' access chambers (see text) to compare the ovary phenotypes of two *Orco* mutants to wildtype (*Ore-R*) and background (w^{1118}) controls upon exposure to queen pheromone. **(A)** The differential response to QMP is shown first via confocal images of *Drosophila* ovaries stained with DAPI. Control genotypes have relatively large, well-developed ovaries that regress upon exposure to [20] qe QMP. The *Orco* mutants, by contrast, have inherently smaller ovaries that are not affected by QMP. **(B)** Bar graphs showing how control lines (*Ore-R*, black, w^{1118} solid grey) respond to QMP, whereas *Orco* mutant lines (*Orco*¹ white, *Orco*² striped) do not. This genotype × treatment effect is significant for both measures of female reproduction (see text). Scale bar = 200 μm.

4.3.2 RNAi knock-down effect on egg number and ovary area

Given the physical (bioassay; Figure 4.2) and genetic (*Orco* screen; Figure 4.3) evidence for olfactory perception of QMP by flies, I used an RNAi screen to identify specific olfactory receptors responsive to the bee pheromone. Of the RNAi lines screened (See Appendix C), a majority had negligible background effects on egg number ($n = 34$ of 45) or ovary area ($n = 26$ of 45). These RNAi constructs are therefore suitable for assessing genuine knock-down effects (i.e., not confounded by background) against QMP within each of these assays.

For egg number, $n = 23$ of the 34 suitable RNAi lines had no appreciable knock-down effect. These genotypes continued to show a worker-like response upon exposure to queen pheromone. The remaining $n = 11$ lines did, however, show a strong knock-down effect, and therefore, do represent candidates for the functional perception of QMP and its downstream effect on egg number (Figure 4.4). The receptors identified from this screen include: *Or9a*, *Or23a*, *Or33a*, *Or43a*, *Or47a*, *Or47b*, *Or49b*, *Or56a*, *Or65b*, *Or85b* and *Or98a*. Of these, *Or49b*, *Or56a*, *Or65b* and *Or98a* had the biggest implied effect as knock-downs were least responsive to the pheromone (Hedge's $g < 0.2$ in all cases).

Likewise for ovary area, $n = 10$ of the 26 suitable RNAi lines had no appreciable knock-down effect and these genotypes continued to show a normal worker-like response to QMP. The remaining $n = 16$ lines did, however, show a strong knock-down that implied a functional role in the perception to QMP and its downstream effect on ovary area. The receptors identified from this screen include: *Or7a*, *Or9a*, *Or22a*, *Or23a*, *Or33a*, *Or43a*, *Or43b*, *Or46a*, *Or47a*, *Or47b*, *Or49b*, *Or56a*, *Or59a*, *Or65b*, *Or85b* and *Or98a*. Of

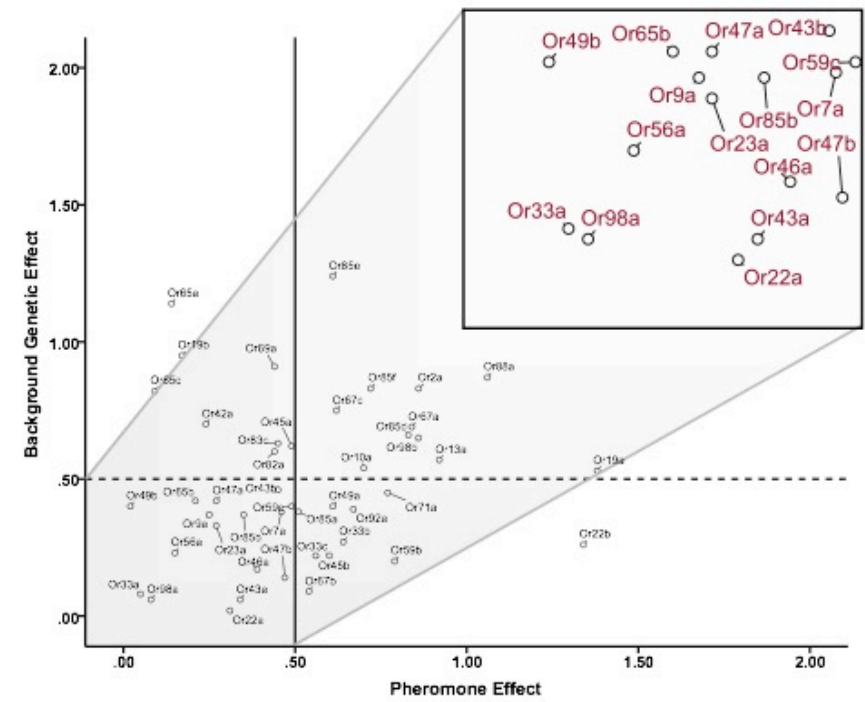
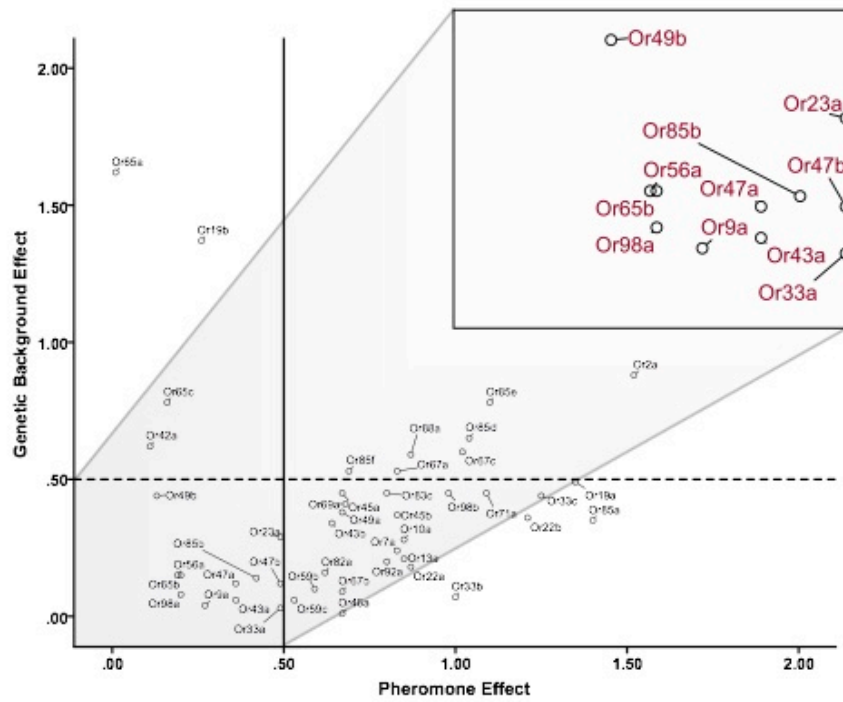


Figure 4.4 Background and treatment effect on ovary phenotypes: a screen of RNAi knock-down lines for olfactory receptors. I assessed the effect of pheromone treatment (x-axis) and genetic background (y-axis) on two measures of female reproductive readiness, egg number (**A**) and ovary area (**B**). To identify specific olfactory receptors (n = 48 screened) that are putatively required for the normal perception of QMP and its downstream effects on ovary phenotype, I measured and plotted the statistical effect size of each olfactory receptor RNAi line, as a function of Hedge's g . I am interested in RNAi lines in the lower left quadrant, and magnified with receptors shown in red, for which the background effect is small ($g < 0.5$ plotted line; that is, knock-down effect itself is not confounded by its background) and pheromone treatment effect is small ($g < 0.5$ plotted lines; knock-down is effective at preventing response to QMP).

these, *Or33a*, *Or49b*, *Or56a* and *Or98a* had the biggest implied effect as knock-downs were, as above, the least responsive to pheromone (Hedge's $g < 0.2$ in all cases).

4.3.3 Predicting the affinity of olfactory receptors to QMP

I compared the sub-structure of receptor ligands to the five individual components of QMP. First, I identified a total of $n = 21$ ligands corresponding to the 16 receptors identified from my RNAi screen. The structural similarity scores between ligand and QMP component ranged from 0.20 – 0.83, suggesting that sub-structural analysis contains ample variation to predict biological affinity. The single most similar receptor-ligand pairings are as follows: *Or33a* with 9-ODA (MCS Ts = 0.62), *Or46a* with HVA (MCS Ts = 0.67), *Or59a* with *cis* and *trans* 9-HDA (MCS Ts = 0.53; Figure 4.5A) and *Or98a* with HOB (MCS Ts = 0.83).

4.4 Discussion

In this study I utilize a novel assay developed by Camiletti et al. (2013) to monitor the affect of a social pheromone on the reproductive physiology of female insects. I expose *Drosophila melanogaster* to a synthetic version of the queen mandibular pheromone of *Apis mellifera* and monitor its effect on fly ovaries. Virgin flies exposed to pheromone develop fewer eggs with smaller ovaries, a response that is comparable to its normal effect on worker bees. That is, it renders them functionally sterile. Second, I show that this response is – at least in part – mediated through olfactory channels, and that knock-down of specific olfactory receptors (e.g., *Or33a*, *Or49b*, *Or56a*, *Or65b* and *Or98a*) can have a big affect on the fly's reproductive response to QMP. Finally, the ligands for two of these receptors – *Or33a* and *Or98a* – are structurally similar to 9-ODA and HOB,

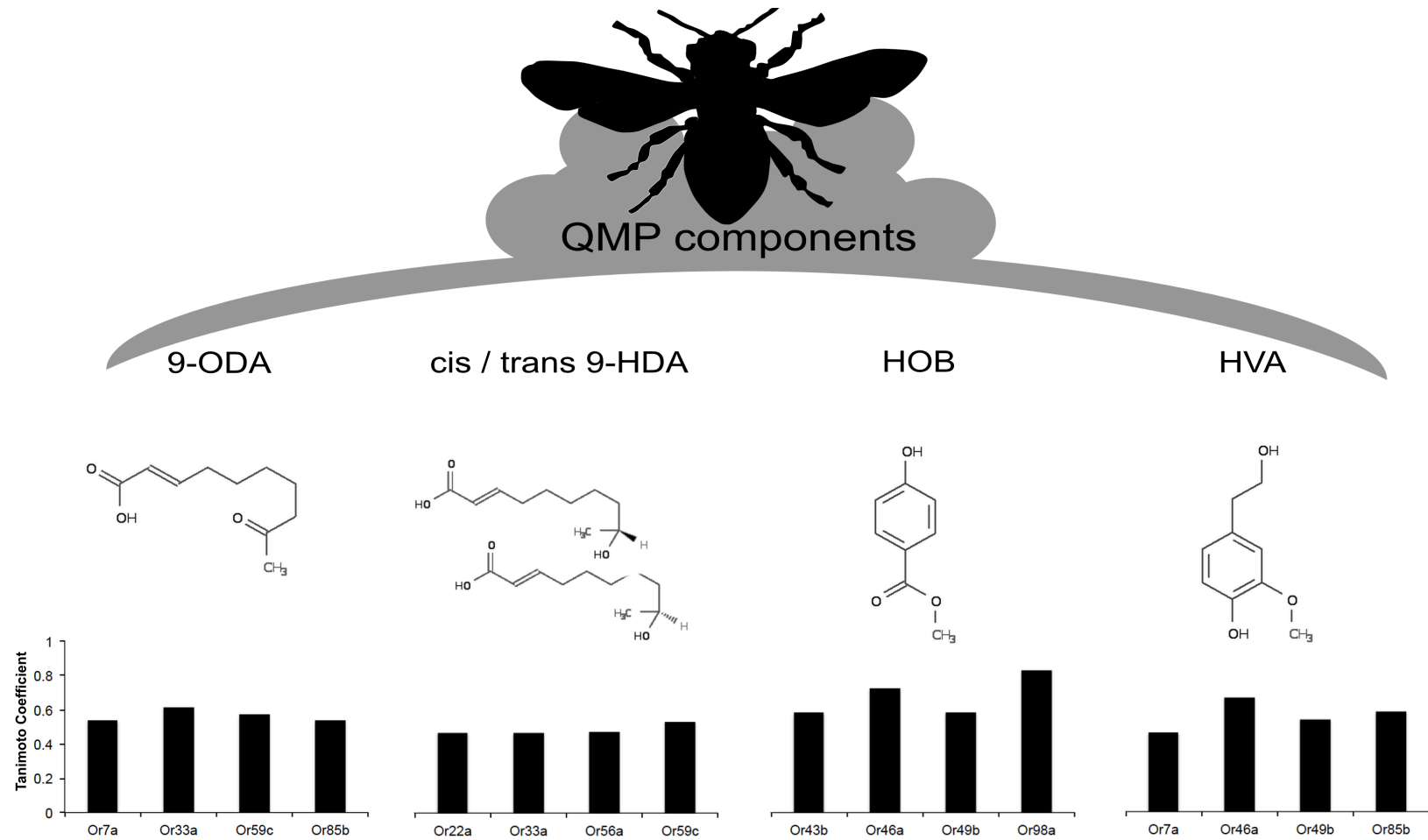


Figure 4.5 Olfactory receptor ligands and their similarity to components of queen pheromone. Queen mandibular pheromone consists of five organic components (9-ODA, cis / trans 9-HDA, HOB and HVA) that show sub-structural similarity to the principle ligands of *Drosophila* olfactory receptors. I used this similarity to identify the receptors that are most likely to be receptive to specific components of QMP, and collectively which sets of receptors are mostly likely to be receptive to QMP as a whole. Bar graphs highlight the olfactory receptors whose ligands have the highest similarity to any one of the five QMP components.

suggesting that the fly's response to bee pheromone may lie in conserved olfactory signaling. It remains to be seen how widely conserved this response is between other social and non-social taxa but my results highlight the utility of *Drosophila* – a genetically tractable but non-social insect – as an adjunct in socio-genetic research. Finally, to the extent that the fly receptors identified here are homologous to those from *Apis mellifera*, they represent the first candidates for the most upstream components of the QMP-mediated pathway that regulates honey bee worker sterility. This finding is significant because the evolution of reproductive altruism must involve indirect selection on specific genes (Hamilton 1964), but the precise genes and pathways are mostly unknown (Thompson et al. 2013).

In this functional study, I show that Dipteran female insects (the *Oregon-R* strain of *Drosophila melanogaster*) respond to social Hymenopteran pheromone that is normally used to regulate worker reproduction within a social context (Hoover et al. 2003; Pankiw 2004). This response from flies to synthetic queen mandibular pheromone (or components thereof) is not unknown; it has previously been recorded from house flies (Nayer 1963; Sannasi 1969; 1972). Together these observations suggest that the queen substance has a strong non-specific effect on the reproductive potential of female insects. A systematic survey is so-far lacking but it appears the ovary inhibiting effect may extend to other social (Carlisle & Butler 1956; Hrdý et al. 1960) and even non-insect arthropods (Carlisle & Butler 1956).

In this study, I determined that the ovary inhibiting effect of QMP is at least partly mediated through near-distance olfaction. When flies are exposed at a distance of ~4 cm

from the source of the pheromone, they de-activate their ovaries to a degree that is comparable to females in full access chambers. This unique observation from female *Drosophila* suggests that the inhibitory effect operates similarly to the situation typical of female bees; workers respond to QMP close to the queen source but this effect diminishes over just centimeters (Kaminski et al. 1990; Katzav-Gozansky et al. 2004). It would be interesting to now compare male flies and bees, where the latter (drones) can clearly detect plumes of QMP over much larger mating flight distances (Gary 1962; Wanner et al. 2007).

Why does the bee pheromone affect the fly or any other non-specific target? First, the response is likely maladaptive. In *Drosophila* populations the forfeiture of personal reproduction is not coupled to alloparental care, and there is no potential for indirect fitness. Flies would also never normally be exposed to social bee pheromone, and thus, the worker-like response from female *Drosophila* [or *Musca* (Nayer 1963), *Formica* (Carlisle & Butler 1956) or *Kaloterme*s (Hrdý et al. 1960)] suggests a common mechanism for de-activating ovaries that is inadvertently triggered by the application of pheromone. Therefore, although *Drosophila* and *Musca* lack any caste-based divisions in labour, there is apparently developmental potential to produce a sub-fertile female in some Dipteran insects that can be induced through QMP. This result is reminiscent of the ancestral developmental potential observed for *Pheidole* ants that can be induced through application of non-specific, synthetic hormone to produce supersoldiers (a type of sub-fertile female) in species that otherwise lack them (Rajakumar et al. 2012). In this case, I suggest that the ability to regulate individual female fecundity in response to environmental cues is deeply conserved in social and non-social insects, as predicted by

West-Eberhard (1996) and others (Hunt & Amdam 2005). The repeated evolution of sub-fertile castes (estimated at between 12-15 times; Bourke 2011; Fischman et al. 2011) may therefore be explained by repeated selection on a common regulatory pathway. If so, I am justified in using tractable but non-social models like *Drosophila* to uncover the pathway.

The hidden developmental potential of conserved genomes is becoming well understood (Carroll et al. 2001), including in the context of social evolution (West-Eberhard 2003). Myself and others (Nayer 1963; Sannasi 1969; 1972), have established the potential for a non-social insect to respond to an ovary-inhibiting social insect cue. One possibility is that QMP is functionally linked to pathways that simply regulate reproduction (Bloch & Grozinger 2011). If so, components of QMP may inadvertently trigger the anonymous pathway. For example, the HVA component of QMP is structurally similar to dopamine (Beggs et al. 2007), and HVA may therefore affect female reproduction through the dopaminergic pathway in bees (Beggs et al. 2007; Vergoz et al. 2012) as it does in non-social insects (Neckameyer 1996). Further, other pathways including the epidermal growth factor receptor (EGFR) signaling pathway (Formesyn et al. 2014), the insulin/insulin-like signaling (IIS) pathway (Mullen et al. 2014; Wheeler et al. 2006), the target of rapamycin (TOR) pathway (Mutti et al. 2011; Patel et al. 2007) and the juvenile hormone (JH) synthesis pathway (Robinson et al. 1992) have all been implicated in the conditional expression of *Apis* worker sterility. These same five pathways related to dopamine (Neckameyer 1996), EGFR (Poulton & Deng 2006), IIS (Badisco et al. 2013), TOR (LaFever et al. 2010) and JH (Riddiford 2012) may likewise function to regulate ovary de-activation in *Drosophila*.

For the top candidates identified from my screen, I compared the biochemical structure of their predicted ligands to the five components of QMP. The ligand for *Or98a* has the highest similarity to any component of QMP. It shows a very high ($T_s = 0.83$) similarity score to methyl p-hydroxybenzoate (HOB), and may therefore have a structural affinity for this component of QMP. The *Or98a* receptor and the HOB component have both been linked to reproduction - for example, *Or98a* mediates female mating behaviour in female fruit flies (Sakurai et al. 2013), and HOB is elevated in mated queens relative to virgins (Plettner et al. 1997). Further, *Or98a* may interact with *spin* and other components of the TOR pathway (Sakurai et al. 2013), which further implicates TOR (Mullen et al 2014). Curiously, *spin* mutants are also characterized by reduced rates of oviposition (Nakano et al. 2001).

Whatever the pathway, olfaction is likely to play a role and potentially provides one relevant environmental cue. In my populations, both *Orco*¹ and *Orco*² had inherently smaller ovaries (approximately 20% smaller relative to background controls), suggesting that olfaction in general is necessary to support full ovary development (Libert et al. 2007). Moreover, the *Orco* mutant's ovaries showed no further reduction upon exposure to QMP. This predicted lack-of-response suggests that the olfactory co-factor *Orco* is specifically required for perception of QMP by flies, as it is in male (Wanner et al. 2007) and female (this study) *Apis mellifera*. The *Orco* gene is unusual among olfactory receptors in its 1-to-1 orthology among insect orders (Larsson et al. 2004). Its relation to the *Apis* genome is therefore clear, and I predict that *AmOr2* is important for the perception of ovary-inhibiting pheromone by workers within queenright hives (Figure 6).

The "tuning" receptors are more difficult to characterize. First, all-but-one of these five receptors (*Or65ba*) are expressed in the basiconic sensilla (Fishilevich & Vosshall 2005) and, beyond QMP, are individually responsive to human sweat (Ray et al. 2014), mold (Stensmyr et al. 2012) or fruit (Mansourian & Stensmyr 2015). At least one of these receptors, *Or98a*, is also functional in sexual signaling (Sakurai et al. 2013). *Or65b* is, by contrast, expressed in the trichoid sensilla, a class of sensory structures specifically responsive to pheromones. The whole olfactory receptor gene family is prone to rapid birth-and-death evolution (Robertson & Wanner 2006), which makes orthology difficult to infer. For example, the average amino acid identity between *Drosophila* OR genes and their top BLAST hit against the *Apis mellifera* genome is less than 25% (not shown). Despite this rapid gene divergence the comprehensive Hymenoptera + *Drosophila* genealogy of olfactory receptor genes of Zhou et al. (2012) provides a useful guide.

From my screen, receptors *Or49b* and *Or56a* cluster with Hymenopteran OR gene subfamilies 'B', 'C' 'D' and 'E', of which there are only $n = 8$ *A. mellifera* orthologues. They are *AmOr116*, *AmOr119* and *AmOr68-AmOr73* (Figure 4.6). These eight *AmOr* genes are therefore clear candidates for the QMP-responsive genes that regulate honey bee worker sterility. Likewise, *Drosophila Or33a*, *Or65b* and *Or98a* form a cluster within the genealogy that is itself sister group to the 'B', 'C', 'D' and 'E' families identified above. This latter set of three *Drosophila* receptors therefore re-enforces the same set of Hymenopteran - and, specifically, the same set of *Apis mellifera* - homologues identified above from this region of the tree. This whole set of $n = 8$ *Apis mellifera* genes are,

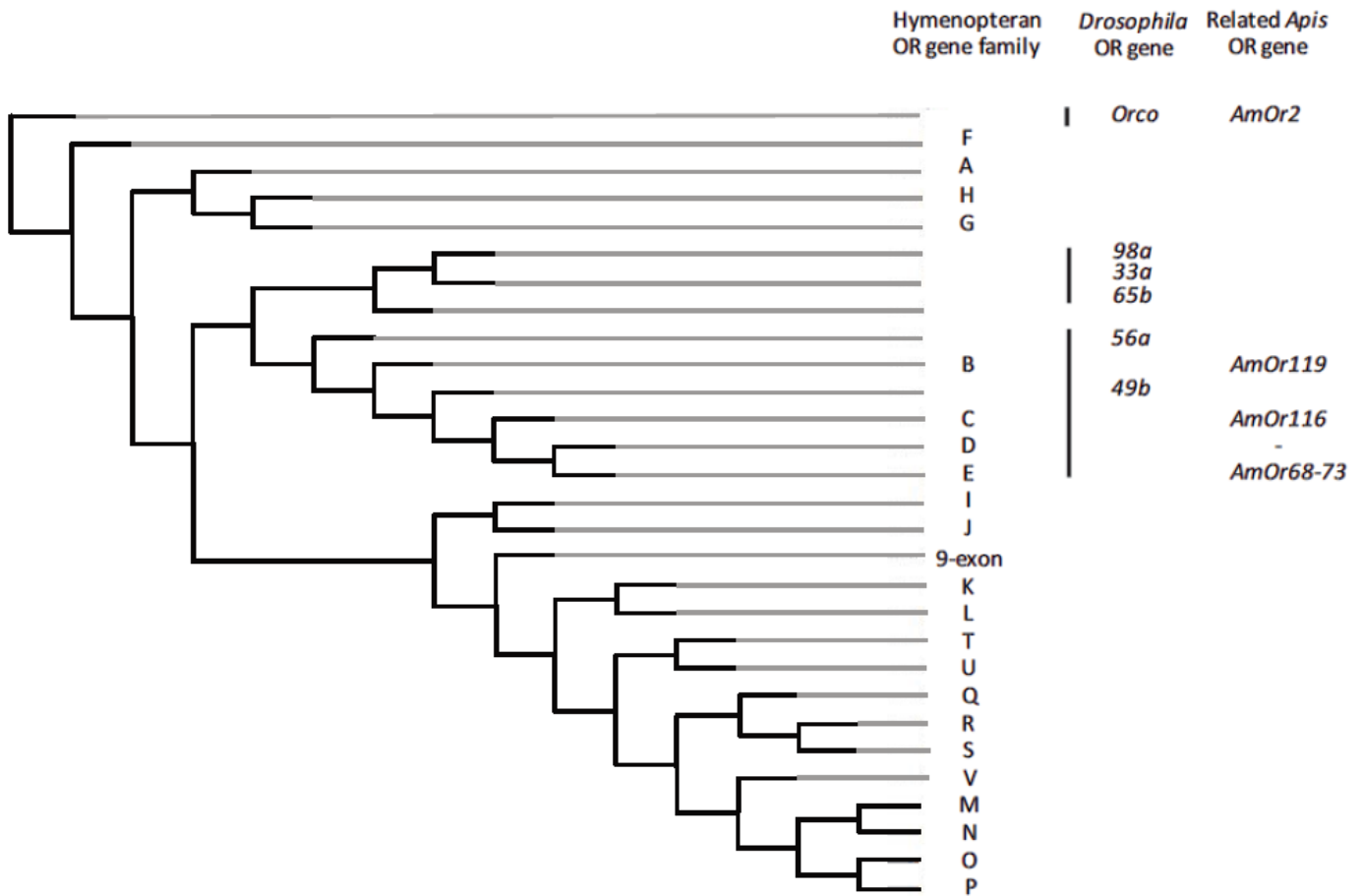


Figure 4.6 Genealogical relationship between Hymenopteran olfactory receptor families (n = 18; A-P, 9-exon, *orco*) and the *Drosophila* olfactory receptors identified from the present screen (n = 6, incl. *orco*). These relationships are re-drawn from Zhou et al. (2012, Supplementary Figure 3 therein) and are here used to identify the most-closely related *Apis* genes (*AmOr*).

therefore, clear candidates for future functional testing. To my knowledge, however, it is not yet feasible to knock-down individual olfactory receptor genes in the sensory tissues of *Apis mellifera* (Jarosch and Moritz 2011). When this functional genomic test is feasible, as it may soon be (Schulte et al. 2014), I can experimentally manipulate the expression of my candidates directly, and measure the response to the ovary-inhibiting pheromone. I predict their individual or collective knock-down will prohibit the worker's stereotypical response to queen pheromone.

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Chapter 5

5 Honey bee royal jelly and queen pheromone as manipulative agents in fly reproduction.

5.1 Introduction

One of the most striking characteristics of eusocial insects is the partitioning of reproduction among females into reproductive and non reproductive specialists. Caste differentiation is for some taxa a function of genotype (Schwander et al. 2010), but most variation in caste is explained by environmental cues (Lattorff & Moritz 2013). For the eusocial honey bee *Apis mellifera*, differences in larval feeding regimes largely explains the differentiation of a genetically totipotent larvae into a reproductive queen or a functionally sterile worker (reviewed by Hartfelder et al. 2015). Within honey bee colonies, female larvae fed almost exclusively on royal jelly develop into queens, whereas, those fed less of this proteinacious substance develop into workers (Haydak 1970). These nutritional differences during early life stages elicit two distinct phenotypic responses; large queens that are long-lived and fecund, and relatively small workers that are short-lived and functionally sterile.

Adult honey bee queens signal their reproductive potential through production of queen mandibular pheromone (QMP). This multi-component pheromone encourages workers to refrain from activating their ovaries and to re-direct their reproductive energy into helping the queen reproduce (Slessor et al. 2005). The queen's signal is proportional to her fertility (Kocher et al. 2008) and declines in fertility are accompanied by changes in

the ratio of components within the pheromone. When queen fertility declines below a threshold level, her daughter workers can to some extent re-activate their ovaries to lay male-haploid eggs.

Queen pheromone and royal jelly therefore have, in a sense, 'opposite' effects on reproduction. Royal jelly is produced by workers and queen caste development. QMP, by contrast, is produced by queens and suppresses reproduction in developed workers. It is not clear, however, if QMP and royal jelly are functionally associated, as regulators of antagonistic pathways or if they simply have functionally opposite effects upon the same regulatory pathway.

Evidence for the later hypothesis suggests that queen pheromone increases the production of the egg yolk precursor protein vitellogenin (Vg) (Fischer & Grozinger 2008), where Vg binds to receptors on the hyperpharyngeal glands, and is subsequently incorporated into the royal jelly medium that nurses feed to developing larvae (Amdam et al. 2003). Further, royal jelly stimulates ovary development (Haydak 1970), and differences in the number of ovarioles (units that produce eggs) within ovaries have been correlated with the threshold response by workers, as workers with more ovarioles are less responsive to QMP (Kocher et al. 2010).

Gene expression studies comparing differences between queen and worker castes and between queen-less (colonies without a queen) and queen-right (colonies with a queen) workers have highlighted the degree of overlap in the genes and gene pathways that regulate caste differentiation and worker ovary activation (Grozinger et al. 2007). The insulin signaling/ IGF-1 like signaling (IIS) pathway regulates queen worker caste

differentiation (Wheeler et al. 2006), as larvae with reduced expression of an insulin receptor, transition into workers even when fed a queen diet of royal jelly (Wolschin et al. 2011). The IIS pathway may also regulate QMP responsiveness as nurses who are more responsive to QMP (Fussnecker et al. 2011), have lower levels of insulin signaling (Mutti et al. 2011b), compared to more QMP unresponsive foragers. Further, juvenile hormone (JH) a key regulator of reproductive development, is up-regulated in queen destined larvae in response to royal jelly, leading to an increased production of Vg that is incorporated into the developing ovary (Barchuk et al. 2002). In adult honey bees, differential expression of JH and Vg are also associated with transitions from nurse and foraging states, and JH specifically is thought to be differentially regulated by QMP component 9-ODA (Robinson et al. 1992).

A comparative study using *Drosophila melanogaster* and *Apis mellifera* has shown that royalactin, the main component of royal jelly, functions as a ligand for the epidermal growth factor receptor (EGFR) in both species. Royalactin-induced increases in EGFR signaling appear to encourage queen development in bees, and to promote queen bee-like characteristics in the fruit fly (Kamakura 2011). In *Drosophila*, royalactin up regulates JH and Vg, inducing a queen-like increase in ovarian development. More recently, it has been shown that decreasing EGFR signaling through genetic knock-down inhibits worker bees from re-activating their ovaries in queen-less environments (Formesyn et al. 2014), suggesting that this pathway may also play a key role in QMP perception. It has already been shown that *Drosophila* females elicit a homologous ovarian response to QMP as seen in worker bees (Camiletti et al. 2013), allowing for a unique approach to test the functional interaction between QMP and royal jelly.

Given the degree of mechanistic relatedness between royal jelly and QMP, it is puzzling why the queen remains fertile in spite of her own production of ovary suppressing queen pheromone. Although this has not been studied in *Apis*, queens of one termite species (Yamamoto & Matsuura 2011) and two ant species (Holman et al. 2012; Vargo 1992) show slight declines in reproduction in response to species-specific synthetic queen pheromone. These responses by queens to their own pheromonal cue are not as dramatic as those seen in workers and suggest that caste specific developmental regimes, like a larval diet of royal jelly, may confer some degree of reproductive safe-guarding to the negative effects of QMP.

In the present study I will conduct two functional tests of the relationship between ovary-inhibiting queen pheromone and ovary-stimulating royal jelly. First, I will measure the ovary phenotypes of egg number, ovary size and fecundity associated with exposing *Drosophila* females to QMP, royal jelly, or both. If QMP and RJ functionally interact to regulate ovary development then I expect, that females treated with RJ and QMP will have a less severe ovary phenotype compared to females treated with QMP alone.

Conversely, if ovarian effect of QMP is not dependent on treatment with RJ, then I expect that the ovarian effect associated with QMP will not be affected by RJ treatment.

In a second test of functional association, I will test the ovarian phenotypes associated with QMP in flies where the effects of RJ have been genetically mimicked, either through up-regulation of the EGFR signaling pathway or ubiquitous expression of royalactin, a key component of RJ. Here again, I expect that if QMP and RJ are functionally associated than I should see less severe ovarian phenotypes in these transgenic flies, than if QMP and RJ were working independently.

5.1 Methods

5.1.1 5.2.1 Fly rearing and housing

I reared strains of *Drosophila melanogaster* on a standard medium of yeast, sugar, agar, and cornmeal, and housed them in a 25°C insect growth chamber (Caron, Marietta, OH) running a 12:12 light: dark cycle at 60% humidity. I placed groups of approximately $n = 30$ males and $n = 30$ females in 60mm collection cages (Diamed, Mississauga, Canada) fitted with plates containing grape juice and agar. I changed plates every 12 hours and left larvae to hatch for an additional 24 hours. I then placed groups of $n = 30$ larvae in vials (28.5 x 95mm, VWR International, Radnor, PA) with food. Finally, I collected same-aged virgin females approximately 10 days later.

5.1.2 Transgenic lines

All royal jelly feeding assays were conducted using wild type *Oregon-R* flies. EGFR overexpression lines included an activated form of Ras oncogene 85D, *UAS-Ras85D* (Karim & Rubin 1998) and the EGFR ligand Vein, *UAS-Vn* (Donaldson et al. 2004). *Elav-gal4* (BID: 8765) was used to express both, *UAS-Ras85D* and *UAS-Vn* lines in the nervous system. For *royalactin* expression studies, I crossed $w^{1118}; +/CyO; UAS-$ *Royalactin* (Kamakura 2011) females with $yw; Actin-Gal4/ CyO$ males (BID: 4144) to generate progeny of $y w/w^{1118}; Actin-Gal4/+; UAS-Royalactin/+$ that had ubiquitous expression of *royalactin*. I crossed w^{1118} (BID: 60000) females with $y w; Actin-Gal4/CyO; +/+$ males, and $w^{1118}; +/CyO; UAS-Royalactin$ females with $y w$ males to achieve control lines of $y w/w^{1118}; Actin-Gal4/+$, and $y w/w^{1118}; +/+; UAS-Royalactin/+$, respectively.

5.1.3 Royal jelly exposure

I transferred solidified standard food medium to a large ZipLock bag and weighed. I purchased two batches of royal jelly from Dave's Apiaries (London, Canada), the first in 2010 and the second in 2014. In both cases I warmed royal jelly in a water bath set to 40°C, and poured it into the standard food medium at a ratio of 20% w/w. I then kneaded the royal jelly/ food mixture by hand in the plastic bag before pipetting 4-5 mL into clean wide food vials. As a control, I used the standard food without royal jelly of the same volume. Finally, I collected eggs, laid three hours apart, from grape juice plates and transferred them into food with royal jelly, or into the control food medium.

5.1.4 QMP exposure

Queen mandibular pheromone is available as a synthetic compound (Contech, Victoria, Canada) that can be diluted from a 500mg stock with 100% ethanol into concentrations of 'queen equivalent' units (qe's) from ~ [3] to [20] qe's by weight. I exposed groups of n=5 same age virgin females to the pheromone by placing them into a modified 50mL Falcon tube that contained a piece of filter paper (grade 413: VWR International, Radnar, PA) with either 20µl of a dose of QMP or a control dose of 20µl of 100% ethanol (as described in Camiletti et al. 2013). Females were housed within these chambers for 48 hours at which point their ovaries were either dissected or they were mated for progeny egg counts.

5.1.5 Ovary scoring

Following QMP exposure ovaries were dissected in 1X Dulbecco's phosphate-buffered saline (1x D-PBS; Invitrogen, Carlsbad, CA) and fixed with 4% paraformaldehyde in D-PBS for 20 min. Ovaries were washed in 1x DPBS and 0.5% PBT (Triton X in 1x DPBS)

stained with DAPI (1:2000) and mounted in 7% glycerol in D-PBS. Ovaries that underwent more in depth egg stage analysis were fanned out using forceps prior to mounting. Ovary specimens were visualized using a Zeiss LSM 5 Duo Vario confocal microscope (Zeiss, Oberkochen, Germany). Number of ovarioles, and number of eggs in each of stages 8 through 14 were scored according to King (1970). Ovary area (μm^2) was estimated using a thresholding calculation implemented in Imaris Pro Plus 7.0 software (Media Cybernetics, Bethesda MD). Ovary area was not estimated for damaged or poorly stained ovaries.

5.1.6 Progeny egg counts

I poured a grape juice and agar medium into the lids of modified Falcon tubes and left to solidify. I then spread a thin layer of yeast paste (yeast and water) onto the middle of the grape juice. Groups of nine flies were placed into these vials and left to lay on the grape juice for 24 hours at a time. Groups of flies consisted of three females that had been reared with or without royal jelly and that had been treated with a dose of QMP, and six same aged males. Eggs were counted after 24 hours.

5.1.7 Fly weight and length measurements

Following QMP treatment, females were CO₂ narcotized and placed into an Epindorf tube and weighed on a microbalance (MX5, Mettler Toledo, Columbus Ohio) in groups of 4 or 5. I then divided the average group weight to give an average weight per individual. I also photographed all flies using an Olympus S7X7 stereomicroscope (Olympus, Richmond Hill, Canada) fitted with Leica KL 300 LED light source and MC170 HD camera (Leica, Wetzlar Germany). From these photographed images, I measured the length of fly heads, thoracies and abdomens using the perimeter

measurement function in Image J (National Institutes of Health). These lengths were then added to give a single total length per fly.

5.1.8 Statistical Analysis

I used an analysis of variance (ANOVA) to assess differences between exposed QMP or pre-treated royal jelly flies for measures of ovary egg numbers and area, progeny counts, and fly body measurements. All statistics were performed using IBM Statistics SPSS 22 (Armonk, NY, USA).

5.2 Results

5.2.1 Effect of royal jelly on adult QMP response.

Drosophila female larvae fed a food medium that contained royal jelly had ovaries that contained more mature eggs ($F_{1,95} = 18.88$, $P < 0.001$) and were larger ($F_{1,95} = 5.46$, $P < 0.05$) than those not fed RJ, regardless of QMP treatment (Figure 5.1, A and B). Although both types of females had a reduction in egg number ($F_{4,95} = 10.25$, $P < 0.001$) and ovary area ($F_{4,95} = 9.13$, $P < 0.001$) in response to QMP, RJ females appeared to be proportionally less effected.

RJ females also laid significantly more eggs following QMP exposure than those not exposed. Exposure to increasing concentrations of QMP reduced the number of eggs laid by females in their first 24 hours following pheromone exposure ($F_{4,76} = 8.66$, $P < 0.001$; Figure 5.2, A). However, this effect was reduced in females that had been pre-fed royal jelly ($F_{1,76} = 53.264$, $P < 0.001$). After 48 hours following pheromone exposure, females

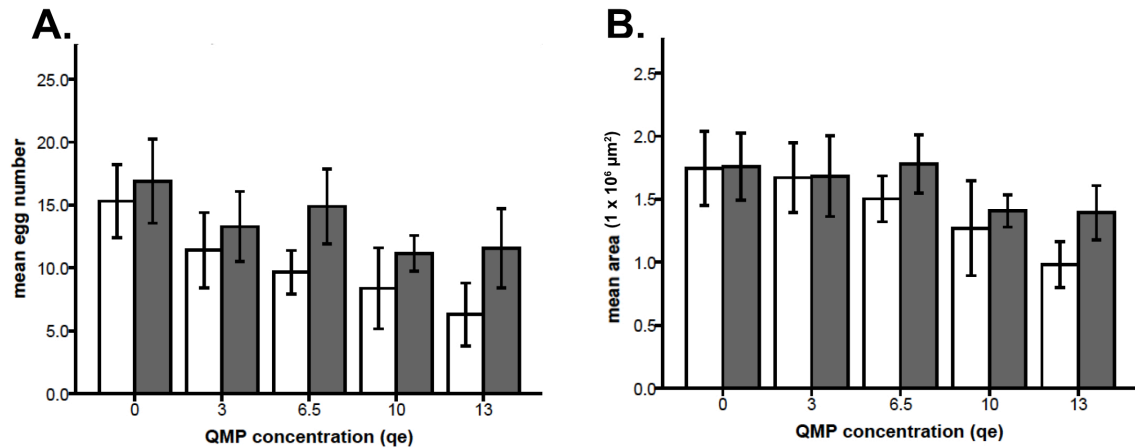


Figure 5.1 Effect of QMP on ovary egg number and ovary area in females supplemented with royal jelly. Egg number (A) and ovary area (B) of females who were exposed to QMP when reared on standard food (white bars) or food supplemented with royal jelly (gray bars). Both response variables (ovary egg number and area) are slightly higher under the royal jelly treated condition. Error bars indicate 95% confidence intervals.

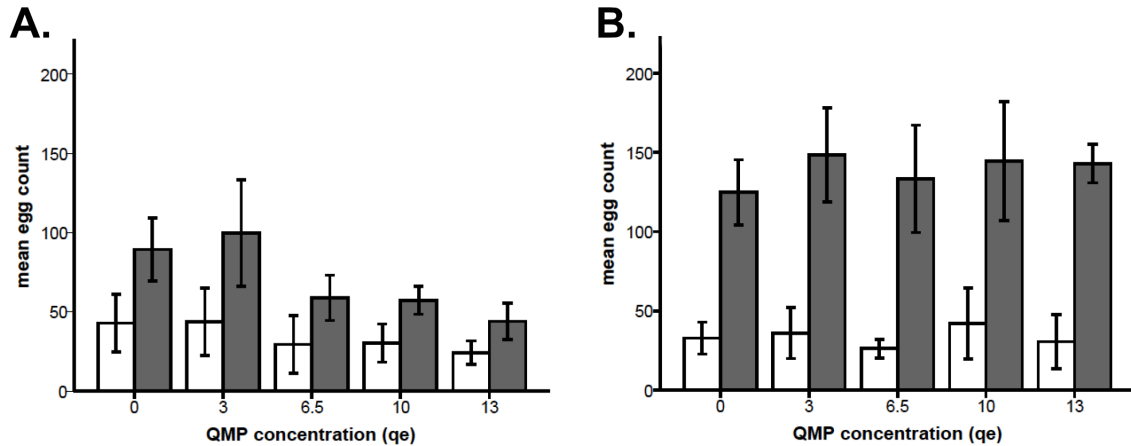


Figure 5.2 Effect of QMP on number of eggs laid in females supplemented with royal jelly. Mean egg counts measured after 24 hours (A) and 48 hours (B) for groups of 3 females who were exposed to QMP when reared on standard food (white bars) or food supplemented with royal jelly (gray bars). Females pre-fed royal jelly laid significantly more eggs than those on a standard diet. Both types of females experienced declines in egg number in accordance with QMP concentration in the first 24 hours. After 48 hours, females appeared unaffected by QMP exposure, yet those pre-fed royal jelly had a higher number of eggs laid overall. Error bars indicate 95% confidence intervals.

were no longer effected by increasing concentrations of the pheromone ($F_{4,76} = 1.00$, $P = 0.42$; Figure 5.2, B), while those pre-fed RJ maintained a large increase in the number of eggs laid ($F_{1,76} = 300.96$, $P < 0.001$).

5.2.2 Effects of royal jelly and QMP on egg stages using different RJ batch

Females pre-exposed to RJ had slightly fewer ovarioles ($M = 34.58$, $SD = 5.54$) than those not exposed ($M = 37.05$, $SD = 3.95$; $F_{1,71} = 5.90$, $P < .018$), yet experienced no differences in other egg stages (8 through 14) measured ($P = 0.24 - 0.81$, Figure 5.3, A and B).

Regardless of RJ pre-feeding, females exposed to either concentration of QMP had a significant reduction in mature egg number compared to females not exposed to the pheromone ($F_{2,71} = 109.24$, $P < 0.001$).

5.2.3 Up-regulation of EGFR and QMP exposure

To mimic molecular effects of royal jelly exposure in females, I up-regulated two components of the EGFR signaling pathway and measured response to queen pheromone. For both egg number ($F_{8,276} = 8.12$, $P < 0.001$) and ovary area ($F_{8,271} = 4.12$, $P < 0.001$), I found significant QMP dose by strain interaction effects, whereby the effect of QMP was dependent upon the fly strain used. Under control conditions of [0] QMP, expression lines of *elav>Vn* and *elav>Ras85D* had significantly more eggs and larger ovaries than at least one of their respective controls (Figure 5.4). Also, up-regulation of EGFR signaling using *elav>Vn* or *elav>Ras85D* was not sufficient to reduced the ovarian response to QMP for egg number of ovary area at either the [13] or [20] qe dose of the pheromone.

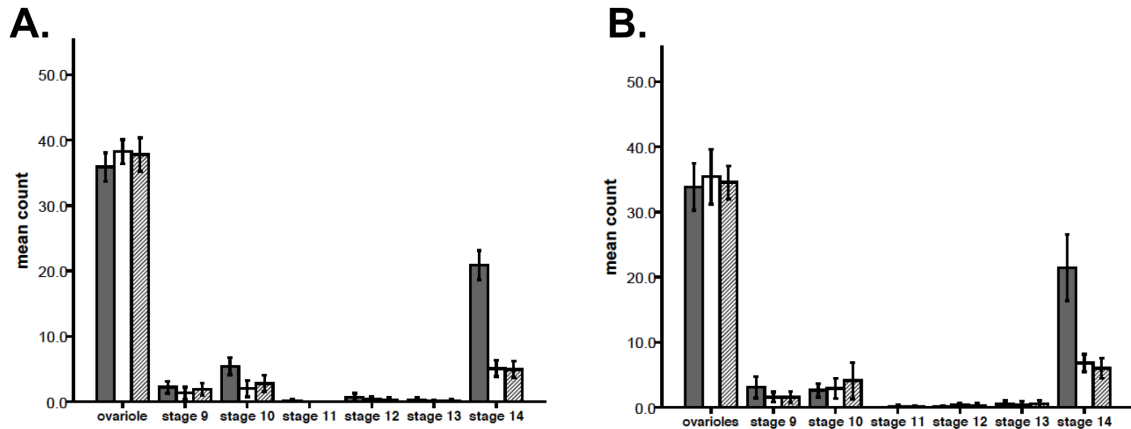


Figure 5.3 Egg stage analysis in females exposed to QMP and royal jelly. Counts of ovary egg stages, ovarioles, and post vitellogenic stages of stage 9 through 14 were counted from pairs of ovaries from females reared on a standard medium (A) and one supplemented with royal jelly (B). Bars indicated concentration of QMP exposure where gray are control doses, white is [13] qe units and striped bars are [20] qe units. Royal jelly had no effect on the number of ovarioles or egg counts at the different stages. QMP appeared only to decrease the number of stage 14 eggs. Error bars indicate 95% confidence intervals.

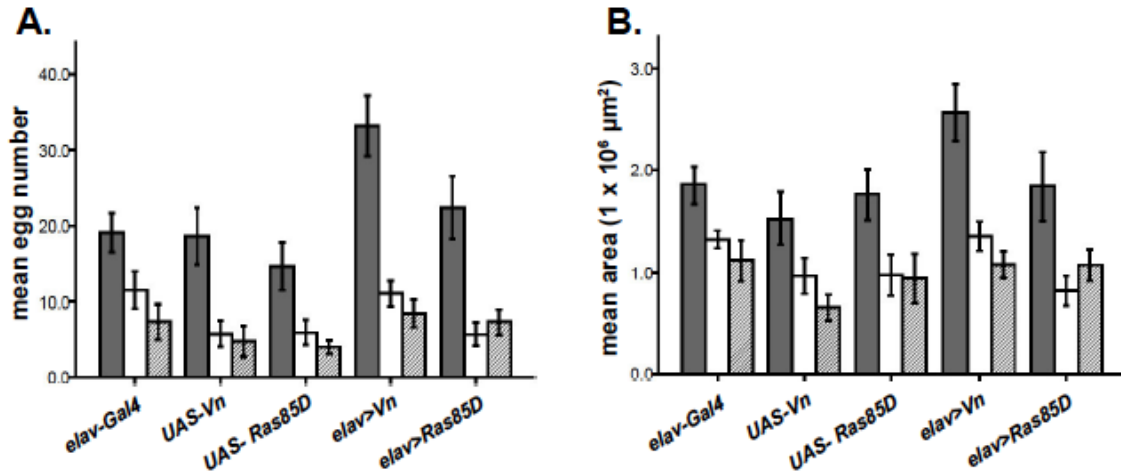


Figure 5.4 Up-regulation of the EGFR signaling pathway on ovarian response to QMP. I compared the effects of QMP exposure, Ovary egg number (A) and ovary area (B), of control lines (*elav-Gal4*, *UAS-Vn* and *UAS-Ras85D*) to those with up-regulated expression of EGFR in the central nervous system (*elav >Vn* and *elav> Ras85D*). Gray bars indicate [0] qe QMP, white bars and striped bars indicate a dose of [13] and [20] qe QMP respectively. Expression of *Vn* and *Ras85D* caused a significant increase in ovary egg number and ovary area compared to controls, however expression did not mitigate the effects to QMP. Error bars indicate 95% confidence intervals.

5.2.4 Ubiquitous expression of royalactin and response to QMP

To determine if royal jelly has any effect on mitigating the ovarian response to QMP, I assessed the response to QMP in flies where *actin*-Gal4 was used to drive expression of UAS-*royalactin*. I found no difference in total fly length ($F_{2, 221} = 0.336$, $P = 0.72$; Table 1) or weight ($F_{2, 45} = 0.367$, $P = 0.70$, Table 2) in *actin* > *royalactin* expression lines compared to controls. I found no significant difference for fly weight ($F_{8, 45} = 2.131$, $P = 0.058$) across the different QMP concentrations or among the different fly strains. Even though there was a significant main effect of QMP dose on fly length ($F_{8, 221} = 3.269$, $P < 0.002$), there were no differences in body length between treated and control QMP doses within any three of the fly lines analyzed. I did; however, found a main effect of QMP ($F_{2, 193} = 50.732$, $P < 0.001$) but not strain ($F_{2, 193} = 1.45$, $P = 0.24$) on mature egg number, again suggesting that ubiquitous expression of *royalactin* had no effect on QMP response.

5.3 Discussion

In this study I tested the functional association of two social cues, royal jelly and queen pheromone. These ovary-affecting agents normally function to regulate caste differentiation and worker sterility in *Apis mellifera*. Using *Drosophila melanogaster* as a model I show that pre-exposure to royal jelly mitigates the ovary-inhibiting effects of queen pheromone. *Drosophila* females fed royal jelly had more eggs and larger ovaries than did females exposed to QMP alone. Moreover, females exposed to both RJ and QMP had higher realized fecundities, as measured as the numbers of eggs laid, than females exposed to QMP alone. This pattern suggests that RJ and QMP may function in a similar pathway as pre-treatment with RJ appears to lessen the severity of the ovarian

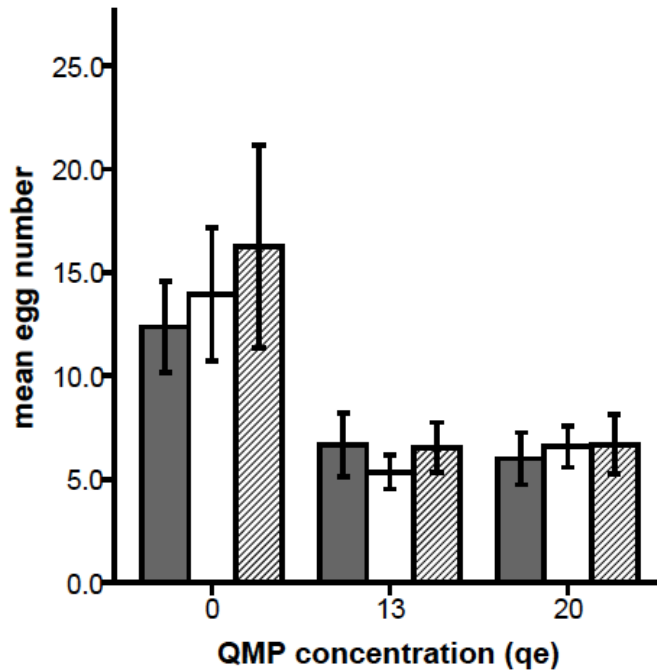


Figure 5.5 Effect QMP on mature egg number in flies expressing *royalactin*. I compared the effects of QMP exposure, ovary egg number, between control lines, *actin-Gal4* (gray bars), *UAS-royalactin* (white bars) to lines with ubiquitous expression of *royalactin*, *actin>royalactin* (striped bars). There was no difference in the effect of QMP ovary egg number across all three lines. Error bars indicate 95% confidence intervals.

phenotype associated with QMP exposure. One caveat to my experiment was, however, that this response was inconsistent. Minor variation in experimental protocol between trails yielded a mix of positive and negative results, suggesting that pathways for reproductive regulation are sensitive to properties of royal jelly itself, ie its quality or composition. I also found that up-regulation of components of the EGFR signaling pathway in neurons was able to mimic the ovarian effect seen with RJ, whereby females had more mature eggs and larger ovaries than controls. This effect however was not able to reduce the effect of QMP, suggesting that the EGFR expression in neurons may not regulate response to QMP. Further I was unable to repeat the effects of Kamakura (2011), and found that ubiquitous expression of a key component of RJ was unable to induce a queen-like response in *Drosophila*, and did not mitigate the ovarian effects associated with QMP exposure.

Initially, I found that *Drosophila* females reared on a royal jelly medium had larger ovaries with more mature eggs than females exposed to QMP alone. This seems to suggest that pre-treatment with royal jelly may reduce the ovary inactivation phenotype seen in QMP-treated females. It is not clear whether QMP and royal jelly function in opposing pathways, or if they may work antagonistically in the same pathway. Evidence for the latter comes from *Apis* where it is suggested that pathways that regulate queen-worker caste differentiation, initiated by royal jelly treatment, highly overlap with pathways involved in worker ovary inactivation, initiated by QMP perception (Grozier et al. 2007). For example, IIS (Mullen et al. 2014; Wheeler et al. 2006), TOR (Patel et al. 2007) and the EGFR signaling pathway (Formesyn et al. 2014; Kamakura 2011) have been implicated in modulating reproductive caste differences between queens and

workers, and in suppressing reproductive activities in workers. It is known that royalactin, the main component of royal jelly, acts as a ligand for the EGFR signaling pathway in *Drosophila* (Kamakura 2011), whereby up-regulation of this pathway induces “queen-like” morphology of increased size and longevity and enhanced reproductive output, measured as the number of eggs laid (Kamakura 2011). I did not find, however, that *Drosophila* females treated with royal jelly alone had an increase in ovary egg number or area under control QMP conditions, suggesting that the effect I observed is more subtle than previously reported.

I was able to successfully repeat the findings of Kamakura (2011) in that females pre-treated with royal jelly were able to lay significantly more eggs during their first 48 hrs after mating compared to females not reared on a medium of royal jelly. This is consistent with the observable effect of royal jelly on ovary size and egg number as the rate of egg deposition and ovary development are normally linked (Bloch Qazi et al. 2003). Secondly, I found that females only experienced a reduction in fecundity in response to QMP up to 24 hours following exposure, and that they laid the same number of eggs as controls after 48 hours. This suggests that the effect of QMP is only temporary as females are able to revert their fecundity when QMP is removed from the environment. This effect is similar to that seen in worker bees, who upon removal of the queen or the synthetic pheromone are able to reactivate their ovaries (Miller & Ratnieks 2001). Although their reproductive potential never reaches the level of the queen, they are able to regain enough functionality to produce male haploid offspring (Bortolotti & Costa 2014).

This reproductive response to royal jelly appears to be sensitive to changes in the quality or composition of the medium used, as changes in batches of royal jelly resulted in different ovarian effects than I found previously. It has been shown that royal jelly proteins are highly sensitive to degradation after collection (Shen et al. 2015); thus, it is possible that my new batch had different nutrient quality or composition of ingredients compared to my initial batch. In light of this, I decided to quantify more discrete egg stages to increase the sensitivity of my measure. Here I found that adults pre-treated with royal jelly had slightly fewer ovarioles than those not pre-treated. This is surprising as honeybee larvae fed royal jelly develop more ovarioles (ovary egg producing units) than controls (Kamakura 2011). Likewise queens, reared almost exclusively on royal jelly, develop up to 180 ovarioles, while workers only develop around 10 (Jackson et al. 2011). This difference in ovariole number may be the result of the new batch of RJ, as low food quality has been shown to be an important determinant of ovariole number (Hodin & Riddiford 2000). Further Kamakura (2011) found that feeding royal jelly that had been heated to high temperatures to developing *Apis* larvae, lead to reduced ovarian development, body size and increased developmental time in proportion to the degree of degradation. I also found that QMP exposure in adulthood did not impact ovariole number. This is consistent with the nature of *Drosophila* ovary development whereby the number of ovarioles within the ovary is fixed upon adult emergence, thus they would not exhibit plasticity to environmental cues like QMP (Hodin 2009).

To analyze the effect of QMP and royal jelly on ovarian maturation I counted vitellogenic staged oocytes 9 through to mature oocyte stage 14 (King 1970). Vitellogenesis begins when oocytes reach their 8th developmental stage at which point, nutrients in the form of

yolk are deposited into the developing oocyte. I found only that the number of mature eggs (stage 14) was reduced in QMP-treated females compared to controls, consistent with my previously published results (Camiletti et al. 2013). Since I only counted later staged oocytes, it is possible that QMP is arresting oocyte development in previtellogenic stages (from the stem cell germaria through to stage 7). In *Drosophila*, it has been reported that in response to environmental cues such as inadequate adult nutrition, development is arrested at two checkpoints in oocyte development, one corresponding to region 2a/2b of the developing germarium, and later at stage 8 (Drummond-Barbosa & Spradling 2001). For example, *chico* mutants with abnormal insulin signaling have increased cell death at region 2a/2b (Drummond-Barbosa & Spradling 2001). Similarly, worker bees exposed to QMP also experience higher instances of cell death in this region (Tanaka et al. 2006). It would be interesting to count pre-vitellogenic staged oocytes in *Drosophila* after QMP exposure to determine if this pattern of cell cycle arrest is conserved between bees and flies.

Expression of EGFR ligand *Vein* (*Vn*) and an activated form of *Ras* (*Ras85D*) in the nervous system, significantly increased the number of eggs and size of the ovaries in the absence of QMP in expression lines compared to controls. These two ligands were chosen as potential mimics of the effects of royal jelly, which functions to increase EGFR signaling during development (Kamakura 2011). *Vn* is an activator of EGFR during the development of the embryonic central nervous system (Schnepp et al. 1996) and *Ras85D* is an activated form of a downstream EGFR target (Gafuik & Steller 2011). The ability of increased EGFR signaling to increase ovary development in my control lines is consistent with results reported by Kamakura (2011) who reported that *Drosophila* females reared

on the EGFR ligand royalactin, the main component of royal jelly, had a significant increase in fecundity, assessed as the mean number of eggs laid compared to controls. Increased expression of *royalactin* was found to cause an increase in juvenile hormone (JH) synthesis and gene expression of *vitellogenin* (Vg), known regulators of *Drosophila* ovary development (Bownes 1982). My results are consistent with previous reports in suggesting that EGFR signaling is important for oocyte differentiation in *Drosophila* (Eppig 2001).

When these same expression lines were exposed to QMP, adults exhibited typical ovarian phenotypes in response to QMP, ie. reduced mature egg number and ovary area. This suggests that stimulation of the EGFR signaling pathway in neuronal tissues is not sufficient to counter the suppressive effects of QMP. This result is surprising, as it has recently been suggested that the ovary inactivation response seen in worker bees is likely the result of suppression of EGFR signaling (Cardoen et al. 2011), and that down-regulation of EGFR signaling prevents worker bees from activating their ovaries in queen-less environments (Formesyn et al. 2014). In contrast to my results where components of the EGFR pathway were up-regulated solely in neurons, Kamakura (2011) found that EGFR signaling in the fat body was needed to produce the developmental phenotypes associated with royal jelly. Therefore it is possible that tissue-specific pathways, perhaps involving EGFR signaling in the fat body, are necessary to regulate QMP ovarian response. QMP may also be functioning through alternative pathways. IIS and TOR, thought to play a role in mediating worker sterility (Mullen et al. 2014) have been shown to mediate downstream ovarian regulators like JH independent of EGFR signaling (Mutti et al. 2011a). In *Apis*, one component of QMP, 9-ODA is able to

suppress JH synthesis (Robinson et al. 1992), but it is not known if this is a direct or indirect effect (Jarriault & Mercer 2012). Since no one clear pathway for ovary inactivation has been elucidated in bees, it has been suggested that there may be multiple alternative pathways, that converge on single ovarian pathways like those mediated by JH, providing more control of reproductive regulation (Mutti et al. 2011a).

Royal jelly contains between 8 and 9 major royal jelly proteins (Furusawa et al. 2008). One of them, mrjp1 (also referred to as royalactin) is the most abundant form and thought to be predominantly responsible for the associated royal jelly phenotypes (Simúth 2001). Using an *actin* driver I ubiquitously expressed *royalactin* in *Drosophila* females and found that its expression was not sufficient to rescue the reduction in egg number associated with QMP exposure. I subsequently found that these lines also did not show increased body weight or size, inconsistent with the results reported by Kamakura (2011). To rule out contamination of the Gal4/ UAS lines, I conducted parallel controls where I used the *actin-Gal4* driver to express GFP and sequenced the *UAS-royalactin* construct to confirm their authenticity and function (data not shown). Ultimately, I was unable to replicate Kamakura's findings regardless of QMP exposure. It would be necessary to more directly confirm the up-regulation of *royalactin* using a technique like quantitative real time RT-PCR along side phenotypic measures to more directly confirm the results of Kamakura (2011).

Taken together I provide preliminary evidence that shows that royal jelly and QMP functionally interact at the level of ovary development, whereby the presence of both cues appear to mitigate the each others effects. This finding, however, appears to be sensitive to the quality of the royal jelly medium, and should be repeated with RJ that has

been verified for quantity and composition of active components like royalactin. It has been hypothesized that pathways that modulate worker sterility and queen-worker caste differentiation likely involve similar pathways (Grozing et al. 2007), however the nature of this overlap remains to be shown. Even as this present study found that neuronal up-regulation of EGFR signaling had no effect on QMP response, it would be worthwhile to explore the role of the EGFR pathway in other tissue types like the fat body to determine if the interaction between QMP and RJ is tissue specific.

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Chapter 6

6 General Discussion

The goal of this thesis was to use the solitary species *Drosophila melanogaster* as a model to gain insights into the genetic basis of reproductive regulation in eusocial species like *Apis mellifera*. In chapter 2, I demonstrate the novelty and benefits of using *Drosophila* as a model for worker sterility. I show that in response to a social cue, queen mandibular pheromone, female flies exhibit an ovary inactivation response similar to that seen in worker bees, whereby, they show a reduction in ovarian size, mature egg number and decreased fecundity. In chapter 3, I implicate a highly homologous gene, *foraging*, in the ovary inactivation response to QMP. I show that in flies, two polymorphs of the *foraging* gene, referred to as sitters (*for^S*) and rovers (*for^R*), are differentially sensitive to the effects of queen pheromone much like their bee counterparts nurses and foragers, respectively. In chapter 4, I show that *Drosophila* can elicit an ovarian response to QMP through olfaction and we identify candidate olfactory receptors in this response. Lastly, in chapter 5, I use *Drosophila* to explore the relationship between two opposing reproductive cues, QMP and RJ, and examine their effects on ovarian development. In this general discussion, I tie together the results presented in each chapter to propose an overarching mechanism for reproductive regulation in response to queen pheromone. In addition, I suggest experimental modifications and additional assays to clarify and build upon these results, and discuss methods to extend the comparative approach towards bridging the evolutionary gap between the fly and bee.

6.1 Towards a complete mechanism of worker sterility

A pathway for worker sterility is likely to be fronted by olfactory receptors, as workers can readily regulate their ovarian development in the presence or absence of a queen pheromonal cue (Bloch & Grozinger 2011). As such, we used an RNAi knock-down screen to identify several candidate ORs that may regulate response to QMP. We suggest that QMP components are preferentially binding to more generalized olfactory receptors that respond to food and environmental odors (Fishilevich & Vosshall 2005), over those normally activated by interspecific pheromonal cues (chapter 4). This is interesting as the insulin/ insulin-like signaling (IIS) pathway, suggested to play a role in worker sterility in *Apis* (Mullen et al. 2014), has been shown to modulate the sensitivity of olfactory receptors required for food acquisition in *Drosophila* (Root et al. 2011). For example, a starvation-induced decline in insulin levels can increase the expression of small neuropeptide F causing increased odor-driven food search behaviours (Root et al. 2011). Further, expression levels of an insulin like peptide, *Drosophila* insulin like peptide 3, is reduced in flies that have a reduced ability to smell (Libert et al. 2007), suggesting that olfaction and the IIS pathway are functionally connected.

Insulin signaling may also regulate QMP responsiveness in worker bees. In *Apis* workers, the transition from QMP responsive nurses to QMP un-responsive foragers (Fussnecker et al. 2011) coincides with a shift in insulin-like peptide expression in the brain and fat body (Ament et al. 2008). The *foraging* gene, responsible for nurse to foraging transition in workers (Ben-Shahar 2003), has been shown to interact with the IIS pathway to modulate fat and carbohydrate metabolism in *Apis* (Wang et al. 2012), and in *Drosophila* (Kent et al. 2009). We also show that *Drosophila* females polymorphic at the *foraging*

gene locus are differentially sensitive to QMP in a manner similar to worker bees (chapter 3), suggesting further that this pathway may modulate QMP sensitivity.

It has been suggested that olfactory receptors that respond to food related cues, like those of the basiconic sensillum, may modulate reproductive success at the level of oviposition (Dweck et al. 2013). Further, the top receptor identified in our screen (Chapter 4), *Or98a*, also of this sensilla type, has an additional role in female receptivity to male advances for courtship (Sakurai et al. 2013). It is important to mention that the developmental time frame we chose for QMP treatment coincides with a period in which females are becoming increasingly attuned to male mating cues (Villella & Hall 2008), and as a result, may naturally be experiencing heightened sensitivity in receptivity receptors as well. Receptivity receptors present an interesting avenue of QMP co-option in *Apis* as worker bees, who refrain from mating, may instead become attuned to the queen's reproductive advantage (Oi et al. 2015).

Or98a is thought to be associated with the target of rapamycin (TOR) pathway; TOR and IIS have been shown to directly mediate ovarian development (Badisco et al. 2013). Mutants for the gene *spin*, a component of the TOR pathway, have reduced receptivity and rates of oviposition (Nakano et al. 2001), while mutants for the insulin receptor substrate, *Chico*, also show reduced ovary phenotypes (Richard et al. 2005). Thus, mutations in both of these pathways produce ovarian phenotypes similar to those seen in QMP-treated females (chapter 2). Insulin like peptides can also stimulate the production of juvenile hormones (JH) from the corpora allata. For example, diapausing adult *Drosophila* females with low JH and insulin titres (Tatar & Yin 2001), show reductions in ovary size. In most insects including *Drosophila*, JH positively regulates vitellogenin

(Vg) synthesis by the fat body and functions alongside ecdysteroids to mediate Vg uptake into the ovary (Flatt et al. 2005). This pathway is slightly different in *Apis* adult females, whereby JH and Vg are able to suppress each other's expression (Amdam & Omholt 2003). Here low levels of JH in ovary inactivated nurses are suppressed by high levels of Vg in their fat bodies (Amdam et al. 2003); while the transition to foraging is associated with high levels of JH and low levels of Vg. Juvenile hormone has also been shown to be sensitive to QMP, whereby 9-ODA, the main component of QMP, causes a reduction in JH production (Robinson et al. 1992). Interestingly, JH has also been shown to regulate female receptivity in *Drosophila* females, suggesting that it may also function at the level of olfaction towards reproductive type cues (Ringo et al. 1991).

Other pathways that have been implicated in ovary regulation in response to QMP include the dopamine signaling pathway and the epidermal growth factor receptor (EGFR) signaling pathway. The QMP component, HVA, is thought to be a functional agonist to dopamine (Beggs et al. 2005). HVA is thought to suppress dopamine levels in *Apis* worker brains (Beggs et al. 2007) and to regulate ovary inactivation through subsets of dopamine receptors in the ovaries (Vergoz et al. 2012). Dopamine signaling is also essential for proper ovary development in *Drosophila* (Neckameyer 1996) and again may interact with olfactory receptors to modulate female receptivity (Neckameyer 1998). In *Apis* it is not clear if dopamine and JH interact to modulate ovary development (Vergoz et al. 2012), yet dopamine appears to be able to modulate JH production in *Drosophila* (Gruntenko Ncapital Ie et al. 2012). Royalactin, the main component of royal jelly, has been shown to function through the EGFR signaling pathway (Kamakura 2011) to stimulate ovarian development through up-regulation of JH and Vg in both *Drosophila*

and in *Apis*. Recently, it has been shown that *Apis* workers with reduced EGFR signaling are unable to activate their ovaries in queenless environments, suggesting that this pathway is involved in QMP-mediated ovary inactivation (Formesyn et al. 2014). We found that females with increased EGFR signaling in their nervous system still had normal ovarian responses to QMP (chapter), however it is possible that the role of EGFR signaling in QMP response is tissue specific and like royal jelly may function at the level of the fat body.

6.2 Modifications to experimental design

As illustrated, the regulation of worker sterility is highly complex, likely involving many interconnected pathways. It has been suggested that different components of QMP can target different pathways (ie, HVA and dopamine; Beggs et al. 2007), with the synergistic action of all components necessary to produce complete ovarian inactivation (Slessor et al. 1990). As such it may be useful to conduct an analysis of each QMP component individually in *Drosophila*, as a way of determining the full complement of pathways involved in response to QMP.

Alternatively, to better understand the synergistic action of the pheromone, it may be useful to expose flies to different blends of QMP that have different compositions of the pheromonal components. For example, the composition of QMP is different between virgin and mated queens, and workers more readily inactivate their ovaries in response to mated queens (Kocher et al. 2009). If *Drosophila* exhibited greater ovary inactivation in response to mated versus virgin queen pheromonal blends, it would highlight the biological relevance of the response to QMP in contrast to a potential pharmacological effect.

At the level of olfaction, these assays could be coupled with more sensitive techniques like gas chromatography coupled with single sensillum recordings to determine which olfactory sensory neurons generate responses to different QMP ligands (Pellegrino et al. 2010). An NFAT (nuclear factor of activated T-cells) and CaLexA (calcium-dependent nuclear import of LexA) based neuronal labeling method (Masuyama et al. 2012), allows you to trace the neural circuits involved in the perception of stimuli from olfactory receptors to antennal glomeruli and to other brain structures like the mushroom bodies (Kain et al. 2013). These approaches would be beneficial in assessing how ligands function individually and also in verifying the candidate receptors that were identified in the olfaction screen.

In the ovary, more specific labeling techniques would be needed to determine the exact stage of ovarian developmental arrest. For example using antibodies like anti-vasa to label cells of the stem cell germaria (Liang et al. 1994), anti-orb to label nurse cells that are destined to become oocytes (Lantz et al. 1994) and TUNEL labeling to detect fragmented DNA and regions of apoptosis, would assist in identifying if oocytes are dying at specific checkpoints.

6.3 Extending the comparative approach

The recent sequencing of the honey bee (Honeybee Genome Sequencing Consortium 2006) and other social insect genomes (Smith et al. 2011) has accelerated comparative study of social and asocial taxa to reveal unexpected conservation of genes related to reproduction and behaviour (Toth & Robinson 2007). As outlined above, genes regulating ovarian development are highly conserved between *Apis* and *Drosophila* (Khila & Abouheif 2010), suggesting that the pathways that regulate reproduction may

have emerged from selection on genes already present in non-social taxa (Amdam et al. 2006; West-Eberhard 1996). To this end, the results from my study and others (Ben-Shahar 2003; Kamakura 2011; Rajakumar et al. 2012) highlight the relevance and utility in using *Drosophila* as a model for the solitary ancestor of the eusocial *Apis mellifera*.

In general the benefits in using *Drosophila* as a model include their ease of laboratory manipulation, their fully sequenced and thoroughly annotated genome, and the many tools available for genetic manipulation (Lin et al. 2014). It is important to note, however, that *Apis* and *Drosophila* are also highly evolutionarily divergent (Dearden et al. 2006), and it may be worthwhile to conduct parallel investigations in species more closely related to *Apis mellifera*. For example, comparing solitary species of sweat bee (Halictidae; Boesi et al. 2009; Richards & Packer 2010) with the honey bee could highlight additional genes involved in the transition from solitary to social behaviour. Recently, there have been advances in the sequencing of additional bee genomes that represent the full range of solitary to eusocial behaviour (Kapheim et al. 2015; Kocher et al. 2013; Sadd & Barribeau 2015), allowing for correlations between genetic modifications, degree of social complexity and phylogenetic context (Rehan & Toth 2015).

The end goal of comparative approaches like mine should be to ultimately test the functionality of homologous genes implicated in fly, directly in the bee. Although the bee is not as amenable to genetic manipulation as *Drosophila* (see chapter 1), there are some promising developments. Recently *piggyBac*-derived cassettes have been coupled with improved laboratory rearing techniques to allow for greater genetic transformational efficiency and conditional expression of transgenic elements (Schulte et al. 2014). Using

these tools it may soon be possible to introduce more powerful techniques like the clustered regularly interspaced short palindromic repeats (CRISPR, Cas9) (Hale et al. 2012) system into the genome of the honeybee (Ben-Shahar 2014), allowing for precise genomic editing. Further, improved transformation techniques could allow for better integration of RNAi elements, that so-far have had limited success (Jarosch & Moritz 2011). Applying techniques like these to comparative studies is necessary to verify the function of homologous genes, and to also better understand how these conserved genes interact with taxonomically-restricted gene sets (Johnson & Tsutsui 2011). Also, using techniques like these to alter gene regulation, the root cause of dimorphic caste development (Simola et al. 2013), could identify how genetically identical larvae develop into QMP producers (queens) and QMP responders (workers).

Lastly, validating homologous genes in the bee allows for the re-incorporation of the social environmental stimuli that was lacking in solitary insects. QMP is not the only factor that likely contributes to worker sterility in honey bees. For example, brood pheromone, produced by larvae, also cues workers to suppress their own reproductive potential to instead feed developing larvae (Le Conte et al. 2001; Maisonnasse et al. 2010). Worker policing, a behaviour in which workers aggressively monitor and attempt to inhibit the reproduction of other workers, also cause workers to refrain from activating their ovaries (Visser & Dukas 1995). Further response to queen pheromones may be dependent on the chemical characteristics of the social group. This has been shown in ant species, *Odontomachus brunneus*, where response to fertility signals are dependent upon whether an individual shares the same chemical background (ie. is from the same population) with the individual producing the pheromone (Smith et al. 2015). Thus the

interaction between genetics and social context may be an important component of worker sterility.

6.4 Conclusion

Inclusive fitness theory explains that altruism persists due to the indirect fitness benefits individuals receive when they forgo their own reproductive potential to assist related individuals. The sequencing of the honeybee genome has accelerated the complementation of this theory with molecular evidence, suggesting that altruistic and social origins arose through co-option of solitary ancestral gene pathways. These ancestral gene pathways, provide the genetic toolkits necessary to expand the social behavioural repertoire, thus increasing social complexity. Comparative studies, like mine, have greatly facilitated the field of sociogenomics, that seeks to explain the genetic basis of social behaviour. My results are the first to use *Drosophila melanogaster* as a model towards social gene discovery for reproductive altruism. In this thesis, I implicate specific genes and gene pathways involved in the regulation of worker sterility, and further highlight olfactory receptors that may be homologous to the immediate early genes involved in QMP response. Taken together I provide direct evidence towards the existence of molecular pathways involved in the reproductive altruistic cascade from perception of cues to ovarian inactivation.

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Authors: Alison L. Camiletti, David N. Awde, Graham J. Thompson

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Appendix C: Chapter 4 Supplemental Information

Supplemental Equation C.1

Effect size comparisons were computed using Hedge's $g = \left[\frac{M1-M2}{SD_{pooled}} \right]$, an unbiased version of Cohen's d index of effect size for sample sizes smaller than $n = 20$ (Hedges, L. V. 1981. Distribution theory for Glass's estimator of effect size and related estimators. *Journal of Educational Statistics*, 6, 107–128), whereby M is the population mean of each response variable, the pooled standard deviation SD of both samples is SD_{pooled}

$$= \sqrt{\frac{(n1-1)SD1^2 + (n2-1)SD2^2}{n1+n2-2}}.$$

Table C.1 A summary of the olfactory RNAi line genotypes (Transgenic RNAi Project #25750) used in the pheromone screen. Shown is the olfactory receptor (OR) common name, the corresponding Gene ID (Flybase), the VDRC ID (Vienna *Drosophila* Resource Center) and the background Library ID from which the transgenic fly was made.

UAS-RNAi Strain (male)					
		Common Name	Gene ID	VDRC ID	Library ID
w ¹¹¹⁸ , elav-Gal4; UAS-Dcr2 (female)	<i>crossed to:</i>	Or85a	CG7454	14099	GD
		Or22a	CG12193	49835	GD
		Or46a	CG17849	4848	GD
		Or67b	CG14176	48511	GD
		Or33b	CG16961	7583	GD
		Or92a	CG17916	36637	GD
		Or47b	CG13206	9354	GD
		Or49a	CG13158	24498	GD
		Or83c	CG15581	42493	GD
		Or67c	CG14156	42710	GD
		Or10a	CG17867	107609	KK
		Or13a	CG12697	104954	KK
		Or19a	CG18859	109388	KK
		Or19b	CG32825	109050	KK
		Or22b	CG4231	105600	KK
		Or23a	CG9880	100866	KK
		Or2a	CG3206	101021	KK
		Or33a	CG16960	101137	KK
		Or33c	CG5006	104198	KK
		Or42a	CG17250	100316	KK
		Or43a	CG1854	110586	KK
		Or43b	CG17853	109777	KK
		Or45a	CG1978	100201	KK
		Or47a	CG13225	100662	KK
		Or49b	CG17584	101293	KK
		Or56a	CG12501	107231	KK

Or59b	CG3569	107460	KK
Or59c	CG17226	101681	KK
Or65a	CG32401	101699	KK
Or65b	CG32402	103959	KK
Or65c	CG32403	106432	KK
Or67a	CG12526	100323	KK
Or69a	CG33264	102981	KK
Or71a	CG17871	110585	KK
Or7a	CG10759	107874	KK
Or82a	CG31519	104532	KK
Or85b	CG11735	106732	KK
Or85d	CG11742	102428	KK
Or85e	CG9700	101916	KK
Or85f	CG16755	108238	KK
Or88a	CG14360	103815	KK
Or98a	CG5540	104155	KK
Or98b	CG1867	101693	KK
Or9a	CG15302	108062	KK

w ¹¹¹⁸	GD CONTROL	60000
y w ;P(attP,y[+],w[3'])	KK CONTROL	601000

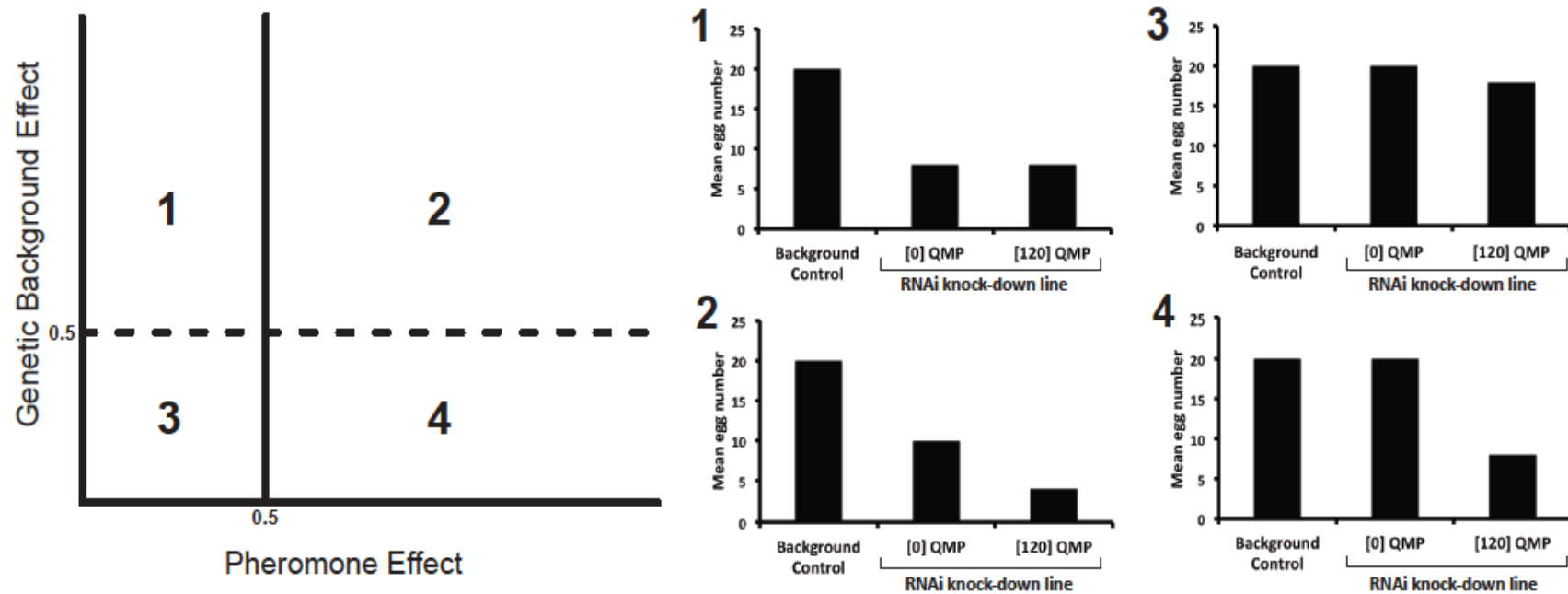


Figure C.1 An illustration of effect size calculations for genetic background and pheromone effects using raw ovary egg number values of hypothetical RNAi lines. Each bar graph illustrates the type of raw values found in each quadrant of the scatter plot from Figure 4.4. For example, in quadrant 1, the genetic background effect size between the background control and the RNAi line in the [0] QMP condition is high, yet the RNAi line shows a small pheromone effect upon treatment with QMP. In quadrant 3, containing my candidate ORs of interest, the genetic background effect size between the background control and the RNAi line at [0] QMP is small, as is the pheromone treatment effect between [0] and [120] QMP.

Table C.2 Hedge's g effect sizes for RNAi lines. Lines shaded dark gray were deemed to have significant background effects - i.e., $g > 0.50$, and were not included in the response-to-pheromone screen. Lines shaded light gray were included in the pheromone screen but did not show a significant knock-down effect - that is, they continued to respond to pheromone treatment, $g > 0.50$. Finally, lines in white represent candidate genes of interest; the RNAi knock-down effectively blocks the worker-like response to pheromone, as evidenced by a statistically small effect on ovary egg number or ovary area ($g < 0.50$).

<i>Hedge's g for ovary egg number</i>			<i>Hedge's g for ovary area</i>		
OR	QMP concentration		OR	QMP concentration	
	[0]	[20]		[0]	[20]
Or65a	1.62	0.01	Or85e	1.24	0.61
Or19b	1.37	0.26	Or65a	1.14	0.14
Or2a	0.88	1.52	Or19b	0.95	0.17
Or65c	0.78	0.16	Or69a	0.91	0.44
Or85e	0.78	1.10	Or88a	0.87	1.06
Or85d	0.65	1.04	Or2a	0.83	0.86
Or42a	0.62	0.11	Or85f	0.83	0.72
Or67c	0.60	1.02	Or65c	0.82	0.09
Or88a	0.59	0.87	Or67c	0.75	0.62
Or85f	0.53	0.69	Or42a	0.70	0.24
Or67a	0.53	0.83	Or67a	0.69	0.84
Or85a	0.35	1.40	Or85d	0.66	0.83
Or19a	0.49	1.35	Or98b	0.65	0.86
Or33c	0.44	1.25	Or83c	0.63	0.45
Or22b	0.36	1.21	Or45a	0.62	0.49
Or71a	0.45	1.09	Or82a	0.60	0.44
Or33b	0.07	1.00	Or13a	0.57	0.92
Or98b	0.45	0.98	Or10a	0.54	0.70
Or22a	0.18	0.87	Or19a	0.53	1.38
Or10a	0.28	0.85	Or22b	0.26	1.34
Or13a	0.21	0.85	Or59b	0.20	0.79
Or7a	0.24	0.83	Or71a	0.45	0.77

Or45b	0.37	0.83
Or92a	0.20	0.80
Or83c	0.45	0.80
Or69a	0.41	0.68
Or49a	0.38	0.67
Or45a	0.45	0.67
Or46a	0.01	0.67
Or67b	0.09	0.67
Or43b	0.34	0.64
Or82a	0.16	0.62
Or59b	0.10	0.59
Or59c	0.06	0.53
Or47b	0.12	0.49
Or23a	0.29	0.49
Or33a	0.03	0.49
Or85b	0.14	0.42
Or43a	0.06	0.36
Or47a	0.12	0.36
Or9a	0.04	0.27
Or65b	0.15	0.20
Or98a	0.08	0.20
Or56a	0.15	0.19
Or49b	0.44	0.13

Or92a	0.39	0.67
Or33b	0.27	0.64
Or49a	0.40	0.61
Or33c	0.22	0.60
Or45b	0.22	0.56
Or67b	0.09	0.54
Or85a	0.38	0.51
Or59c	0.40	0.49
Or47b	0.14	0.47
Or7a	0.38	0.46
Or43b	0.46	0.45
Or46a	0.17	0.39
Or85b	0.37	0.35
Or43a	0.06	0.34
Or22a	0.02	0.31
Or47a	0.42	0.27
Or23a	0.33	0.27
Or9a	0.37	0.25
Or65b	0.42	0.21
Or56a	0.23	0.15
Or98a	0.06	0.08
Or33a	0.08	0.05
Or49b	0.40	0.02

Table C.3 Tanimoto's similarity coefficients for pairwise comparisons of different olfactory receptor ligands and the components of QMP. Shading represents the top four similarity scores for ligands and their corresponding olfactory receptors for each component of QMP. Light grey shading corresponds to ligands that are specific to one component of the pheromone, while dark grey shading represents ligands that were specific to more than one component of QMP.

Receptor	Predicted ligand	<i>cis-/trans-</i>		HOB	HVA
		9-ODA	9-HDA		
Or98a	ethyl benzoate	0.333	0.3158	0.8333	0.4375
Or43b	ethyl trans-2-butenate	0.4	0.375	0.5833	0.4286
Or46a	4-methylphenol	0.4	0.375	0.7273	0.6667
Or49b	2-methylphenol	0.3125	0.2941	0.5833	0.5385
Or33a	2-heptanone	0.6154	0.4667	0.2667	0.25
Or85b	e2-hexenol	0.5385	0.5	0.5	0.5833
Or7a	E2-hexenal	0.5385	0.5	0.3846	0.4615
Or59c	3-octanol	0.5714	0.5333	0.25	0.3125
Or43a	1-hexanol	0.4285	0.5	0.2857	0.3571
Or22a	methyl octanoate	0.5	0.4706	0.375	0.2778
Or43b	ethyl butyrate	0.3125	0.2941	0.4615	0.333
Or47b	gamma butyrolactone	0.3571	0.25	0.4167	0.2857
Or22a	ethyl hexanoate	0.4375	0.4118	0.4	0.2941
Or22a	isobutyl acetate	0.2353	0.2222	0.3571	0.25
Or47a	butyl acetate	0.2353	0.2941	0.3571	0.333
Or85b	6-methyl-5-hepten-2-one	0.4667	0.4375	0.333	0.4
Or47a	pentyl acetate	0.2941	0.3529	0.333	0.3125
Or23a	1-pentanol	0.3571	0.4286	0.3077	0.3846
Or9a	3-hydroxy-2-butanone	0.3571	0.333	0.3077	0.2857
Or43a	cyclohexanol	0.333	0.4	0.2857	0.3571
Or56a	geosmin	0.4211	0.4737	0.1905	0.2381

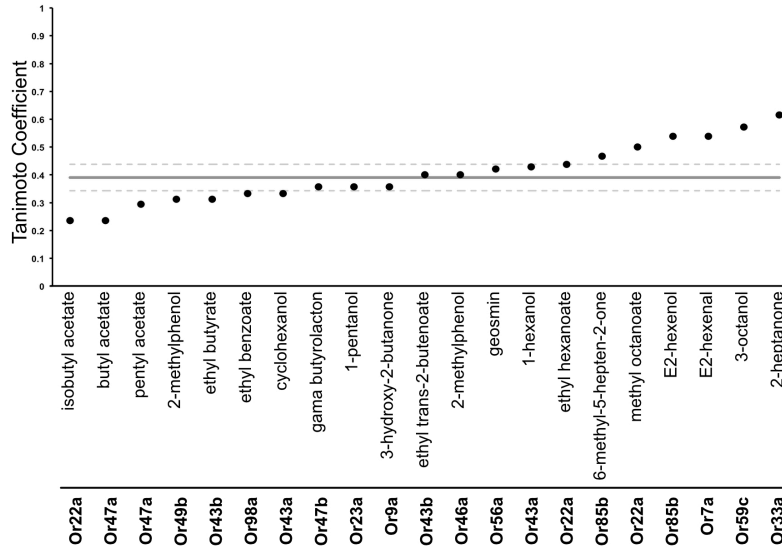
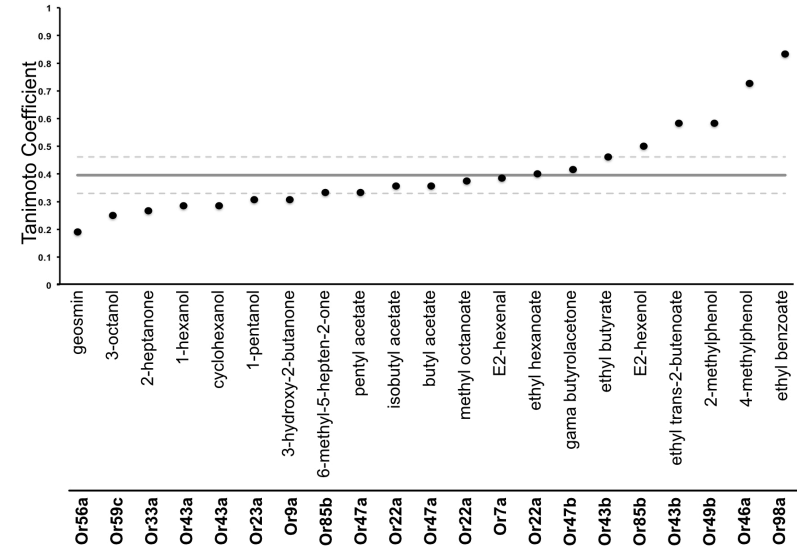
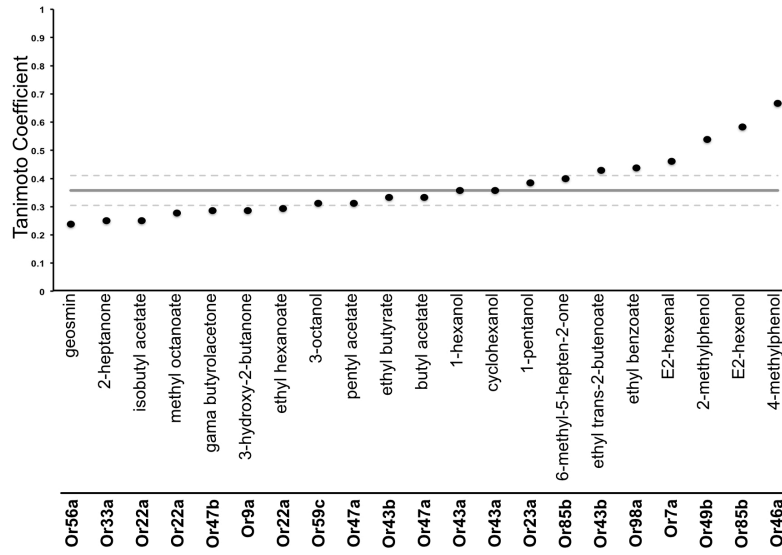
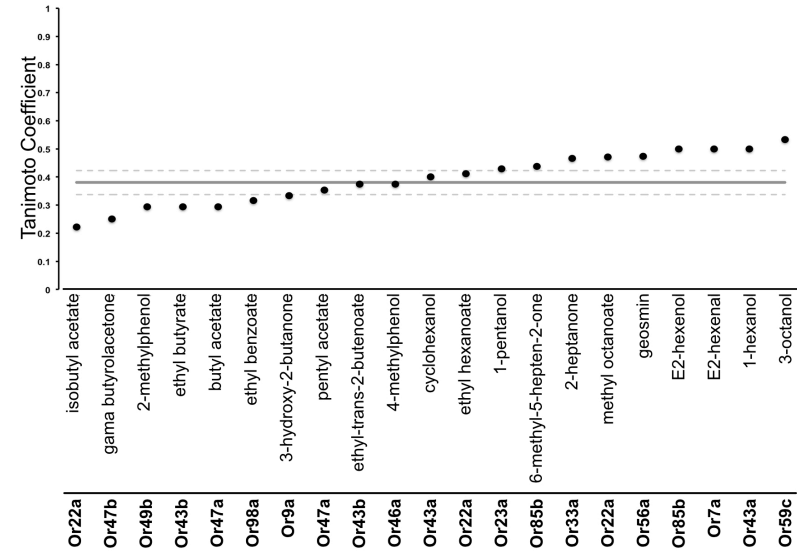
A. 9-ODA**B. HOB****C. HVA****D. cis- and trans- 9-HDA**

Figure C.2. Similarity scores for all 21 ligands (and their respective receptors) are shown against each component of QMP. The average similarity score (with 95% CI) is shown as a solid line (mean MCS Ts = 0.36 to 0.40).

Appendix D. Chapter 5 Supplemental Information

Table D.1 Body measurements of the head, thorax and abdomen for lines with ubiquitous expression of *royalactin* (*actin>royalactin*) and control genotypes (*UAS-royalactin* and *actin-Gal4*) after exposure to different concentrations of QMP as indicated. Total length for measurements added across head, thorax and abdomen are also shown along side the number of flies counted per row (N). All measurements are represented in millimeters as mean +/- SD.

Genotype	QMP	Head	Thorax	Abdomen	Total
<i>actin>royalactin</i>	0	0.47 +/- 0.048	0.98 +/- 0.059	1.35 +/- 0.130	2.80 +/- 0.148, N = 25
	13	0.45 +/- 0.038	0.97 +/- 0.073	1.32 +/- 0.076	2.74 +/- 0.101, N = 24
	20	0.47 +/- 0.035	0.98 +/- 0.043	1.36 +/- 0.094	2.80 +/- 0.124, N = 23
<i>UAS-royalactin</i>	0	0.46 +/- 0.043	0.98 +/- 0.057	1.43 +/- 0.120	2.87 +/- 0.163, N = 25
	13	0.46 +/- 0.039	0.99 +/- 0.045	1.29 +/- 0.092	2.74 +/- 0.098, N = 25
	20	0.48 +/- 0.047	0.97 +/- 0.048	1.30 +/- 0.087	2.76 +/- 0.124, N = 25
<i>actin-GAL4</i>	0	0.47 +/- 0.050	0.99 +/- 0.054	1.36 +/- 0.099	2.80 +/- 0.135, N = 25
	13	0.48 +/- 0.053	0.99 +/- 0.033	1.33 +/- 0.101	2.79 +/- 0.129, N = 24
	20	0.46 +/- 0.044	0.98 +/- 0.070	1.28 +/- 0.105	2.72 +/- 0.155, N = 25

Table D.2 Fly weights for lines with ubiquitous expression of *royalactin* (*actin>royalactin*) and control genotypes (*UAS-royalactin* and *actin-Gal4*) after exposure to different concentrations of QMP as indicated. Weights given as the mean +/- SD weight per fly per genotype in mg units.

Genotype	QMP	Weight
<i>actin>royalactin</i>	0	1.03 +/- 0.150, N = 5
	13	0.93 +/- 0.023, N = 5
	20	0.97 +/- 0.094, N = 5
<i>UAS-royalactin</i>	0	1.09 +/- 0.152, N = 5
	13	0.90 +/- 0.110, N = 5
	20	0.98 +/- 0.132, N = 5
<i>actin-GAL4</i>	0	1.07 +/- 0.107, N = 5
	13	0.97 +/- 0.071, N = 5
	20	0.97 +/- 0.072, N = 5

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May 2010. From non-social to eusocial. A comparative genomic analysis of genes related to reproduction in the fly vs. bee. Cold Spring Harbor Laboratory Meeting on Honey Bee Genomics and Biology, Cold Spring Harbor, New York

March, 2010. The *Drosophila* Ran-Binding Protein in the Microtubule organizing center (*RanBPM*) gene is required in the mushroom bodies neurons for larval behaviour. Women in Science and Engineering (WISE), Hamilton, Ontario

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